The cellular and molecular effects of UVA1 on human skin in vivo Impact on Human Health

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The cellular and molecular effects of UVA1 on human skin *in vivo:* Impact on Human Health

Thesis submitted to the Faculty of Medicine of the University of London for the degree of Doctor of Philosophy

By

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Signed Declaration

I declare that the work presented in this thesis is my own except for the ribonucleic acid (RNA) extraction from whole skin biopsies and microarray hybridisation, which were performed by Milteyni Biotec, Germany

Angela Tewari

Abstract

The incidence of non-melanoma and melanoma is increasing and becoming a greater public health concern. Despite extensive research into the action spectra for the induction of skin cancers, the relative contributions of the different wavebands of ultraviolet radiation (UVR): UVB (290–320 nm), UVA2 (320–340 nm) and UVA1 (340–400 nm) are largely unknown. Until recently, UVA1 was thought to be relatively insignificant despite making up 75% of environmental UVR, as well as being the major spectral region in tanning lamps. High dose UVA1 is also used in phototherapy. Recent work demonstrating UVA1-induced cyclobutane pyrimidine dimers (CPD) *in vitro* and human skin *ex vivo* provides a clear indication to examine its effects *in vivo* in humans. The aims of this thesis was to assess DNA damage and other biological markers, in skin type I/II individuals after environmentally relevant doses of UVA1, and study its genetic effects using whole genome analysis. UVA1 formed CPD in human skin, predominantly in the basal epidermis, in contrast to erythemally equivalent doses of UVB of which these lesions were 3-4 times more frequent and were preferentially located in the upper layers of the epidermis. Previous studies suggest that repair of UVA1 CPD is slower than that induced by UVB. Our studies show that CPD repair kinetics were the same for both UVB and UVA1 when the whole epidermis was examined, however UVA1 CPD were poorly repaired over a 48 hour (h) period when assessment was restricted to the basal layer. We show that this is probably due to an overexpression of basal epidermal p63 by UVA1, and a concomitant lack of apoptosis of damaged cells. Overall, this is likely to make UVA1 CPD more mutagenic, as they persist for longer and are more likely to be incorporated as mutations during replication. Whole genome microarray

studies showed distinct time dependent changes in many cellular pathways, with inflammation through T_H17 signalling being the top upregulated pathway at 6h, and extracellular matrix remodelling being the top upregulated pathway at 24h. We found a dramatic upregulation in MMP12 gene expression by UVA1 at 24h. MMP12 protein is predominantly formed by UVA1 and also exhibits elastolytic activity. Our studies may explain how UVA1 contributes to late solar elastosis characterized by elastin degradation, and clinically by 'sagging' of the skin. MMP12 is a good marker of UVA1 exposure and MMP12 inhibition may be a new therapeutic approach for delaying photoageing and also photocarcinogenesis. To conclude, these results necessitate the long term follow up of patients receiving UVA1 phototherapy and clearer information for public health measures on photoprotection and tanning lamp legislation.

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Abbreviations

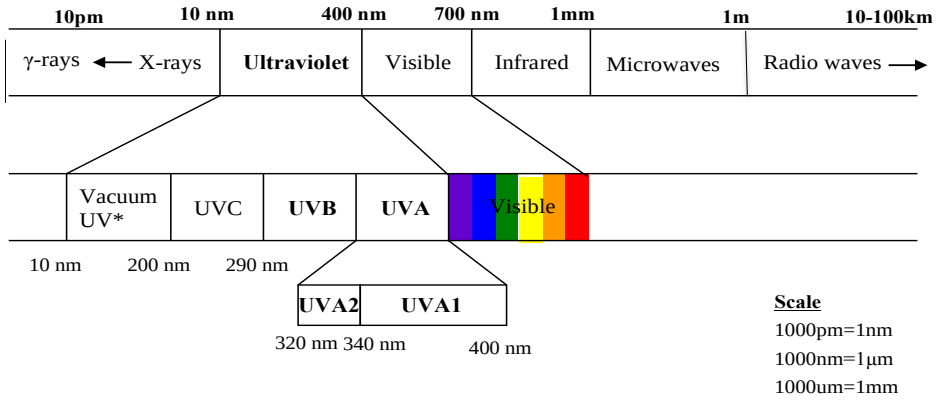
AP1	Activator protein 1
APC	Antigen presenting cell
BCC	Basal cell carcinoma
BER	Base excision repair
CD	Cluster of differentiation
CHS	Contact hypersensitivity
CIE	Commission Internationale de 1'Eclairage
COX2	Cyclooxygenase-2
CPD	Cyclobutane pyrimidine dimer
Cy3	Cyanine 3-labelled
DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DNCB	Dinitrochlorobenzene
DPX	p-xylene-bis-pyridinium bromide
DT	Delayed tanning
DTH	Delayed type hypersensitivity
e-	Electron
e.g.	*exempli gratia*
ECM	Extra cellular matrix
EEE	Erythemally effective energy
EGFR	Epidermal growth factor receptor
EPP	Erythropoietic proporphyria
ERK	Extracellular regulated kinase
FADH	Flavin adenine dinucleotide
FasL	Fas ligand
FES	Feature Extraction Software
GO	Gene ontology
H_2O_2	Hydrogen peroxide
HO1 (HMOX1)	Haemoxygenase 1
i.e.	*id est*
ICAM1	Intercellular adhesion molecule 1
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL	Interleukin
IR	Infrared radiation
IPD	Immediate pigment darkening
IR	Infrared
JNK	c-Jun NH2-terminal kinase
LC	Langerhans cell
MAPK	Mitogen-activated protein kinase

MED	Minimal erythema dose
MHC	Major histocompatibility complex
MM	Malignant melanoma
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NFκB	Nuclear factor kappa light chain enhancer of activated B cells
NK	Natural killer
$O_2•^{-2}$	Superoxide
OH-	Hydroxyl ion
$^1O_2$	Singlet oxygen
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDT	Photodynamic therapy
PGE_2	Prostaglandin E_2
pIX	Protoprophyrin IX
PLE	Polymorphic light eruption
PPD	Persistent pigment darkening
PTCH	Patched
RCT	Randomised control trial
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT	Reverse transcription Polymerase Chain Reaction
RTqPCR	Real time quantitative Polymerase Chain Reaction
SCC	Squamous cell carcinoma
SED	Standard erythemal dose
SEN	Sensitiser
SPF	Sun protection factor
SSR	Solar simulated radiation
T reg	Regulatory T cell
T4N5	T4 endonuclease V
TGFβ	Transforming growth factor β
T_H1	T helper 1
TNF	Tumour necrosis factor
UCA	Urocanic acid
UVA1	Ultraviolet A1
UVB	Ultraviolet B
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
XP	Xeroderma pigmentosum
Λmax	Absorption maximum

Chapter 1 Introduction

1.1 Ultraviolet radiation and the electromagnetic spectrum

Ultraviolet radiation (UVR) forms part of the electromagnetic spectrum: a series of oscillating waves in which the frequency of each wavelength defines the energy it carries. These include radiowaves, microwaves, infrared radiation (IR), visible light, x-rays and gamma radiation (Diffey and Kochevar, 2007) and the shorter the frequency, the greater the energy it carries (see Figure 1.1). UVR (100-400nm) lies between the visible spectrum and the x-ray waveband. Within this, the biological effects of UVR vary considerably with wavelength and for this reason the Commission Internationale de 1'Eclairage (CIE) (Diffey, 2002) has further subdivided UVR into 3 regions: UVC 100-280nm, UVB 280-315nm and UVA 315-400nm. Stratospheric ozone (0_3) effectively absorbs wavelengths shorter than 290nm, and all of UVC and so for the purpose of this thesis its properties shall not be discussed further. As there is significant photobiological activity at wavelengths <320nm, environmental and dermatological photobiologists usually define UVB as 290-320nm and UVA as 320-400nm. The UVA waveband has been subdivided into UVA2 (320-340nm) and UVA1 (340-400nm).



*blocked by air but can travel through a vacuum

Figure 1.1 UVR and the electromagnetic spectrum
Using photobiological wavelengths of UVA (320-340nm) and UVB (290-320nm)

1.1.1 Solar UVR

The spectral distribution of solar energy at sea level comprises roughly 3–7% UVR (290–400 nm), 44% visible light (400–700 nm), and 53% IR (700–1440 nm) (Frederick *et al.*, 1989). Of the ~5% of terrestrial UVR reaching the earth's surface 95-98% is UVA and 2-5% is UVB. UVA1 makes up ~75% of terrestrial UVR. The intensity and spectral composition of solar UVR fluctuates due to a number of environmental factors including the solar zenith angle (which depends on latitude, season and time of day), the stratospheric O_3, atmospheric pollution, and attenuation by cloud cover. For example daily UVR intensity varies throughout the day because as the solar zenith angle increases, the spread of UVR rays emitted by the sun is distributed over a larger area on the earth's surface. Also UVB rays are attenuated more than UVA rays due to absorbance by 0_3 thus whilst UVA remains relatively constant throughout the day (Diffey, 2002; Jansen *et al.*, 2013b) the proportion of UVB changes. Highest UVR intensity occurs between 11am and 3pm (81% of daily UVR (Diffey, 2002)). Cloud cover also reduces UVB values by 25-30% which means that at higher altitudes the intensity of UVR is greater and the spectral distribution may vary (Diffey, 2002).

1.2 Structure of human skin

Human skin is composed of an outer cellular epidermis (~100-150μm) and an inner dermis (~ 2-4mm) below the basement membrane (Figure 1.2). The epidermis contains 3 major resident cell populations: keratinocytes, Langerhans cells (LC) and melanocytes. Keratinocytes are the predominant population and they originate from the actively dividing basal epidermal layer. The process of terminal differentiation,

whereby keratinocytes differentiate and migrate towards the *stratum corneum* is under tight homeostatic control and takes approximately 4 weeks, (Iizuka, 1994; Weinstein *et al.*, 1984) although rates of proliferation and maturation are faster in response to injury, inflammation and disease. LCs function as antigen presenting cells (APCs) and melanins are synthesised by melanocytes, which are also thought to possess immunomodulatory capabilities (Lu *et al.*, 2002). The second layer of the skin, the dermis, is a connective tissue layer composed largely of collagen I and III (Gordon and Hahn, 2010) known as the extracellular matrix (ECM), and resident cells include fibroblasts, dermal dendritic cells and macrophages. The epithelial layer is nourished by the blood vessels in the dermis, which also contains a network of sensory nerve endings responding to touch, temperature and pain including substance P and Calcitonin Gene-related peptide (CGRP).

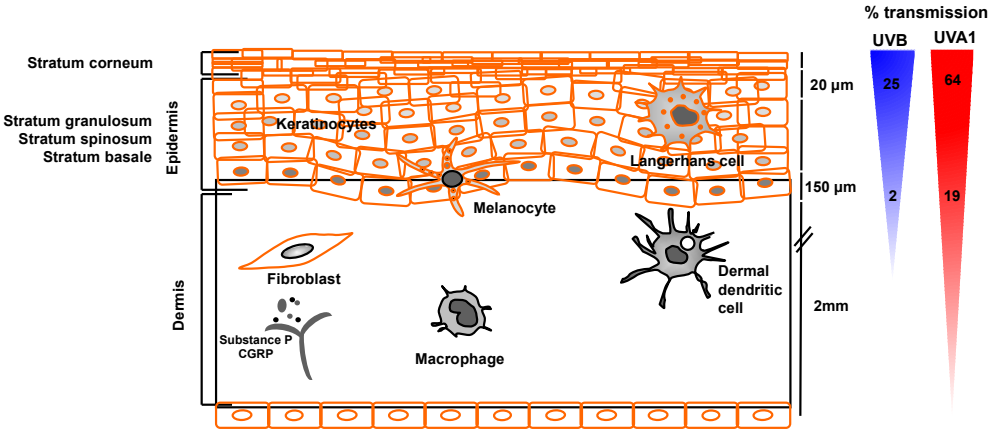


Figure 1.2 Structure of human skin at rest
Ex vivo transmission (Bruls *et al.*, 1984) with UVB at 297nm and UVA1 at 365nm

1.3 UVR transmission through the skin

As UVR reaches the skin, a certain amount is reflected (approximately 4-7% throughout the UVR spectrum) (Anderson and Parrish, 1981). The 93-96% of incident radiation that is not reflected may be absorbed or scattered within any layer

of the skin, which determines the penetration of radiation. Generally there is more transmission, scatter (by molecules smaller than the wavelength) or reflection (by molecules larger than the target wavelength) and dermal remittance (a type of back scatter/reflectance) at longer wavelengths (Anderson and Parrish, 1981; van Gemert *et al.*, 1989) which explains how longer wavelengths penetrate deeper into the skin (Bruls *et al.*, 1984). On reaching the skin, UVR photons are absorbed by target chromophores to initiate a series of biological responses.

1.3.1 Chromophores

Each type of molecule in the skin: nucleic acids, aromatic amino acids (tryptophan, phenylalanine, tyrosine, histidine), melanins (phaeomelanin and eumelanin) absorb a unique combination of wavelengths and are termed chromophores (Diffey and Kochevar, 2007; Young, 1997). The absorption spectrum of a given chromophore is the probability of absorption of photons as a function of wavelength. Pure DNA has an absorption maximum (λmax) at 260nm (UVC), readily absorbs in UVB but weakly absorbs in the UVA waveband (Setlow, 1974; Sutherland and Griffin, 1981). At less than 300nm, aromatic amino acids, nucleic acids, urocanic acid and melanin are the major epidermal absorbers (Figure 1.4) (Anderson and Parrish, 1981). Most chromophores absorb in more than one spectral region for example, NAD+ has an λmax at 260nm and in the reduced form (NADH) absorbs maximally at 339nm (UVA1). Other than melanin, UVA chromophores in the skin include βcarotene, protoporphyrin IX (pIX) and riboflavin. Exogenous UVA chromophores include antibiotics such as tetracyclines and quinolones (Makinen et al., 1997) which can cause drug-induced photosensitivity reactions, and topical/oral psoralens

(furocoumarins) which in combination with UVA form the basis of PUVA phototherapy (Morison and Honigsmann, 2007).

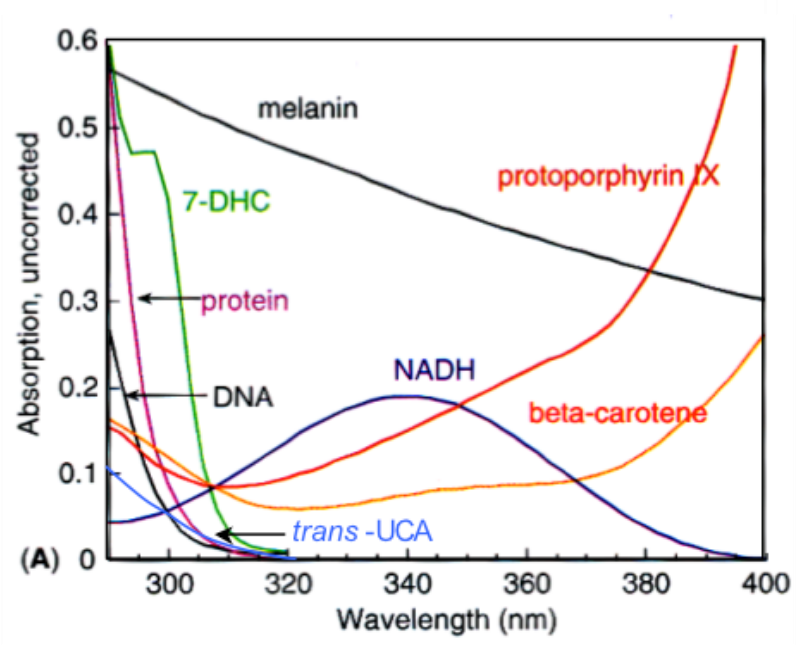


Figure 1.3 Absorption spectra of skin chromophores
Adapted from Diffey and Kochevar (2007)

Energy states and electron transfer

When a molecule or sensitiser (Sen) absorbs a UVR (or visible light) photon it enters an "excited state" (Sen*). This is either an excited singlet state (existing for only a few nanoseconds) and returning to ground state by emitting excess energy as fluorescence or heat (internal conversion), or an excited triplet state which is at a slightly lower energy state and persists for longer. A triplet excited state returns to ground state and gives off energy as phosphorescence. Singlet or triplet states are determined by the electron spins which affects the amount of energy they hold. In a singlet state, the electrons spin in opposite directions and exist at a higher energy level compared with an excited triplet state where the spin of both electrons in the energy shell are in the same direction (Figure 1.4).

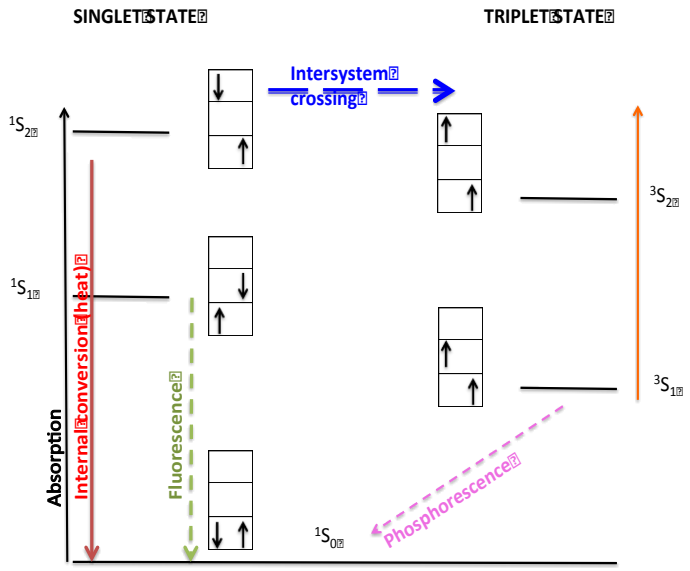


Figure 1.4 Jablonski Diagram
(Energy transitions following absorption of UVR by a photosensitiser)

In both energy states the Sen can undergo chemical reactions (known as either type I or type II photosensitisation). In type I, the excited Sen (which is generally in a triplet state) reacts directly with the substrate. The substrate donates an electron (e-) to create a Sen radical anion (Sen□-) and a substrate radical cation (Substrate□+). Sen□- can then transfer the e- to oxygen producing the superoxide (0_2□-) or hydroxyl (OH□-) radical ions, which also in the process regenerates the sensitiser. In type II photosensitisation the transfer of energy from the photosensitizer to oxygen produces an excited singlet oxygen ($^1O_2$) which can either undergo photochemical reactions (Figure 1.5), or undergo intersystem crossing, where the spin of the electron flips and transition to the lower energy triplet state ($^3O_2$), which persists for longer to cause cellular damage.

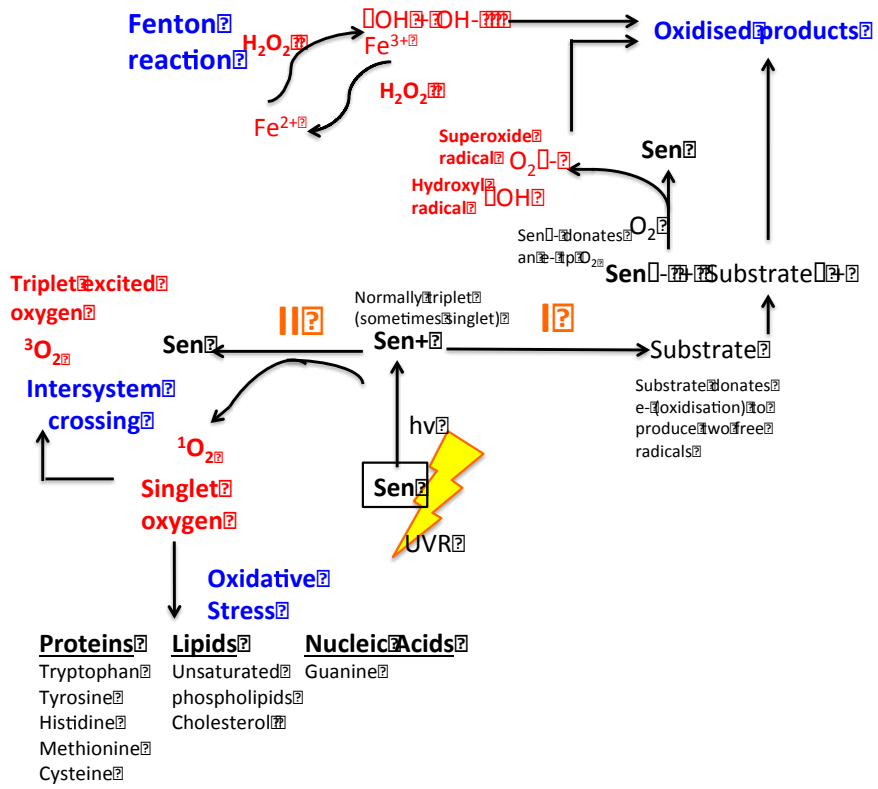


Figure 1.5 Type I and type II photosensitisation reactions
In type I photosensitisation, the energy is transferred to produce an excited sensitiser and in type II the energy is transferred to oxygen leading to free radical production and tissue damage.

Erythropoietic proporphyria (EPP) is an example of a skin disease where clinical symptoms manifest through type II photosensitisation. Due to a metabolic defect, there is an accumulation of the photosensitiser protoporphyrin IX(pIX) in endothelial cells and on absorption of blue light (400-410nm), $^1O_2$ is generated leading to cellular damage and causing the photosensitive symptoms of pain, oedema, necrosis and occasionally erythema. Cytotoxicity through $^1O_2$ also forms the basis of photodynamic therapy (PDT) (Ibbotson and Szeimies, 2007) and UVA1 phototherapy (Krutmann and Morita, 2007) in the treatment of skin conditions.

There are also other electron transfer systems in the skin and one of the most effective oxidisation processes in cellular biology is the Fenton reaction. In the presence of H_2O_2 and 0_2□- as often found in type I photosensitisation these oxidise Fe^{2+} to Fe^{3+} (ferric iron) forming OH□- radicals and hydroxide (OH-) ions. This is a powerful self-perpetuating cycle as Fe^{3+} can be reduced back to Fe^{2+} by another molecule of H_2O_2 and the damage cycle continues (Bickers and Athar, 2006).

1.3.2 Action spectroscopy

An action spectrum is a measure of the wavelength dependency of a given photobiological effect and is plotted as the reciprocal of the dose required to produce a given end-point (y axis) against the wavelength (x axis). This plot is typically constructed by performing dose-response studies with monochromatic radiation. In some cases, the slope of the dose-response curves is used as the index of efficacy.

The CIE erythema action spectrum in humans (Figure 1.6) (CIE, 1998), shows that UVB is 3-4 orders of magnitude more erythemally effective than UVA however these data are variable at wavelengths greater than 340nm. More recently, work examining erythema with lasers (Anders *et al.*, 1995) has identified a further erythemal peak at ~360nm, suggesting two independent mechanisms of erythema induction. Erythema is a marker of DNA damage up to 340nm (Young *et al.*, 1998a) and as DNA is a chromophore for UVB, erythema is likely mediated through direct absorption by DNA whilst UVA is probably absorbed by another unknown chromophores in the skin in the presence of oxygen (Auletta *et al.*, 1986) indirectly resulting in erythema.

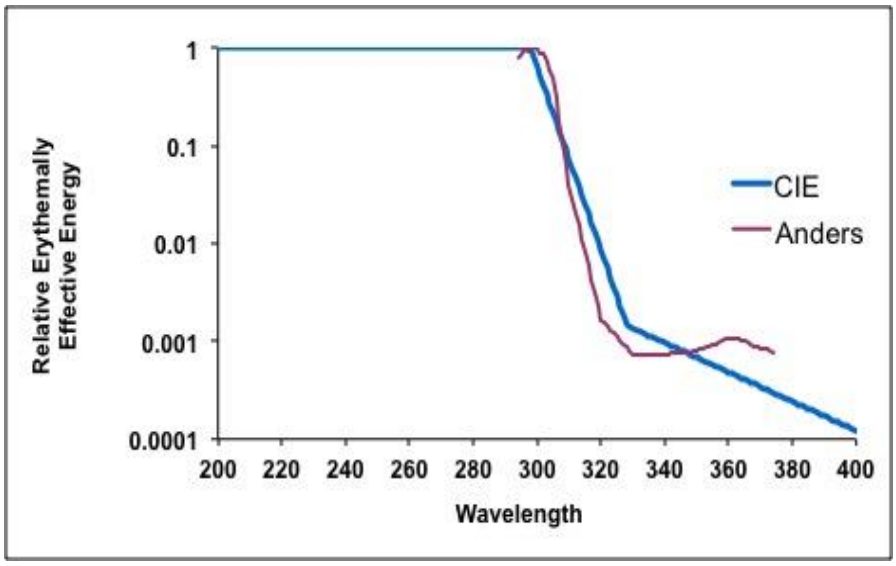


Figure 1.6 Action spectra for erythema
The CIE reference action spectrum for erythema in human skin (blue) and the Anders action spectrum using narrow waveband lasers (red) (Anders *et al.*, 1995; CIE, 1998)

Action spectroscopy has two main functions (i) *chromophore identification:* for example DNA is a chromophore for erythema and also TNFα (Walker and Young, 2007) and (ii) *the determination of biological weighting functions for a given emission spectrum:* e.g. solar UVR where 3% UVB in a given solar simulated spectrum accounts for 65% of erythemally effective energy (EEE) (Young *et al.*, 2010).

1.3.3 Sensitivity of skin to UVR

A system based on self reported susceptibility to sunburn forms the basis of our current skin type I-VI classification (Fitzpatrick, 1988) (Table 1.1). Studies on skin types I-III show an increase in the ratio of epidermal eumelanin (black/brown melanin) to phaeomelanin (reddish brown) through skin type groups I to III (Thody *et al.*, 1991) which suggests eumelanin has photoprotective properties likely acting as a photoprotective cap to keratinocyte DNA (Kobayashi *et al.*, 1998). Individual

skin sensitivity to UVR is assessed by determining the minimal erythema dose (MED); the dose of a given UVR source needed to produce visible erythema. The MED is widely used as an exposure dose unit in phototherapy and experimental photobiology to assess a biological response (Diffey, 2002). As UVB is approximately 1000 fold more effective than UVA per unit physical dose at inducing erythema i.e. at 300nm the dose needed to give a MED is 0.030 J/cm^2 whereas at 360nm this dose is $32J/cm^2$ (Young *et al.*, 1998a), sunburn is often considered to be a UVB dependent response. This is not entirely the case as a broad-spectrum sunscreen provides better protection to cumulative erythema than a UVB sunscreen (Young *et al.*, 2010).

The MED of individuals with skin type IV is about twice that of individuals with skin type I (Harrison and Young, 2002), however there is considerable overlap, thus skin type is not necessarily a reliable indicator of sensitivity to sunburn (Young, 2006). Given that the MED is variable even within a given skin type, the standard erythemal dose (SED) is increasingly used to measure erythemally effective exposure doses (Diffey *et al.*, 1997). It has the advantage of being independent of personal UVR sensitivity and emission spectrum of source. 1SED is equivalent to an erythemally effective exposure dose of $100J/m^2$ (or $10mJ/cm^2$). Table 1.1 classifies human skin according to its constitutive melanin pigmentation, ability to tan in sunlight and typical dose needed to give an MED.

Skin type	Susceptibility to sunburn	Constitutive skin colour	Tanning ability	Susceptibly to skin cancer	No of SED to give an MED
I	High	White	Very poor	High	1-3
II	High	White	Poor	High	
III	Moderate	White	Good	Low	3-7
IV	Low	Olive	Very good	Low	
V	Very low	Brown	Very good	Very low	7->12
VI	Very low	Black	Very good	Very low	

Table 1.1 Classification of skin type I-VI
According to tanning ability and indicative MEDs expressed in SEDs that might be expected following exposure on UVR naïve skin (Diffey and Kochevar, 2007, Harrison and Young, 2002).

1.4 Effects of UVR in the skin

The acute and chronic effects of UVR on the skin are summarised in Table 1.2.

	Acute effects	Chronic effects
280-320nm (UVB)	**Molecular** DNA damage Photoisomerisation of trans-urocanic acid (UCA) **Cellular** Immunomodulation **Clinical** Inflammatory reactions leading to erythema Immunosuppression Delayed tanning Synthesis of Vitamin D	Photocarcinogenesis Photoageing
320-400nm (UVA)	**Molecular** DNA damage **Cellular** Immunomodulation Photoisomerisation of *trans*-urocanic acid (UCA) **Clinical** Inflammatory reactions leading to erythema Immediate pigment darkening Persistent pigment Darkening Immunosuppression	Photocarcinogenesis Photoageing

Table 1.2 Effects of UVR in human skin *in vivo*
Adapted from (Lautenschlager *et al.*, 2007)

1.4.1 Acute effects of UVR on the skin

1.4.1.1 Molecular

DNA damage: CPD, 64PP and Dewar isomer

Peak DNA damage in human skin occurs at 300nm (Young *et al.*, 1998a) due to absorption and diffusion of the shorter wavelengths of UVB in the skin. UVB causes the formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine (64) pyrimidone photoproducts (64PPs), which in the presence of UVA radiation (at 320nm) (Cadet *et al.*, 2005), is converted to its Dewar photoisomer. Action spectroscopy for CPD and 64PP *in vitro* in human skin fibroblasts are similar from 254-302nm although CPDs are induced 5-10 fold more frequently (Rosenstein and Mitchell, 1987).

Dimers form after the absorption of a photon of energy which splits the C5=C6 double bond of two adjacent pyrimidine (thymine (T) or cytosine (C)) bases, and forms new covalent bonds linking pyrimidines at the C5 and C6 positions. A 4-carbon cyclobutane ring is produced, giving the dimer its characteristic name. With 64PP, the C5=C6 double bond breaks and the surplus energy results in the rotation of one of the pyrimidine rings which offers its C4 (of the 3'-end) to form a new bond with the C6 (of the 5'-end) of the adjacent pyrimidine (Ichihashi *et al.*, 2003) (Figure 1.7). In this case only one new covalent bond is formed. This structure causes a more significant distortion in the double helix than the cyclobutane ring, which is thought to cause its more rapid recognition and repair (Mitchell and Nairn, 1989; Young *et al.*, 1996).

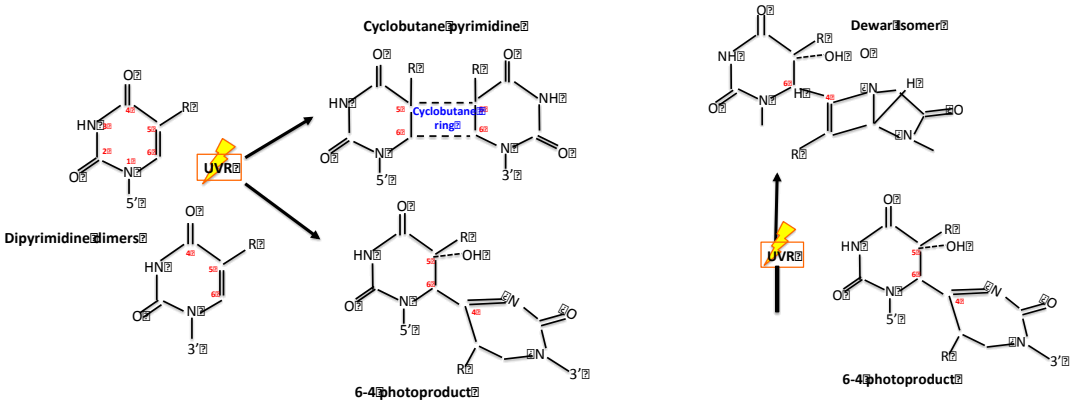


Figure 1.7 DNA damage photoproducts (CPD, 64PP, Dewar isomer)

8oxodG/8oxoGua

Oxidative stress is a state in which the cellular antioxidant system is overwhelmed by reactive oxygen species (ROS) and results in modifications of cellular biomolecules including lipids, proteins and nucleobases. Guanine has a low threshold for oxidation, and as guanosine (where it is attached to a deoxyribose sugar) it forms 8-oxo-7,8-dihydro2deoxguanosine (8oxodG) also known as 8oxodGuo. Its nucleobase equivalent is 8-oxo-7,8-dihydroguanine (8 oxo-guanine) (8-oxoGua) (Figure 1.8).

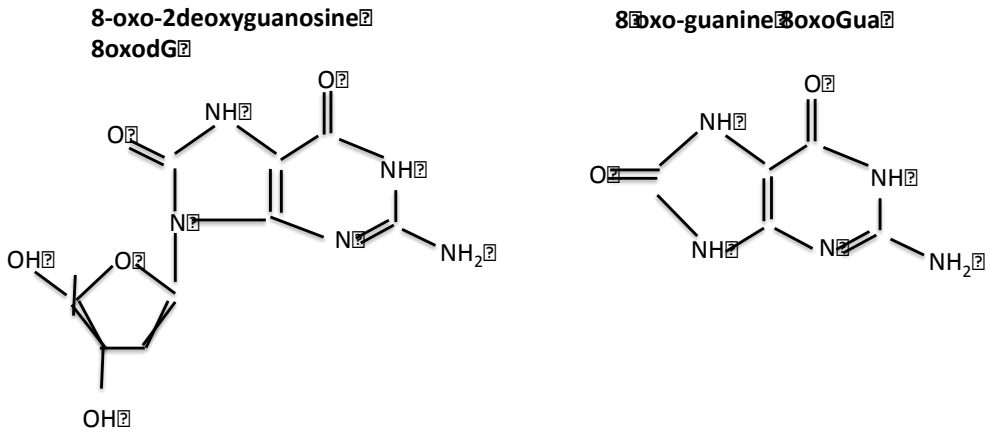


Figure 1.8 8oxodG/8oxoGua

CPDs are responsible for >80% of UVB induced mutations in DNA repair proficient mammalian cells (You *et al.*, 2001). The TT is more persistent than C containing CPD in human skin *in vivo* because of its slower repair (Bykov *et al.*, 1999; Xu *et al.*, 2000). If unrepaired, these interfere with base pairing during DNA replication. Briefly, C containing dimers are not appropriately recognised and instead of G, an A is placed opposite. This means that in subsequent replication cycles T is placed opposite A leading to a C→T transition (known as the C:G→A:T transition) or a tandem CC→TT transition. These were typically known as "UVB signature mutations" as they were almost exclusively thought to be due to UVB (Brash *et al.*, 1987; Drobetsky *et al.*, 1987). Cytosine containing dimers are thought to be most mutagenic (Douki, 2013; Mouret *et al.*, 2008)) as the mutation frequency recovered at dipyrimidine sites in UVR irradiated cells generally contain cytosine (Pfeifer *et al.*, 2005). Bulky TT dimers are repaired by the "A rule" which is where A is placed opposite appropriately due to bypass at the replication fork by DNA polymerase η otherwise known as bypass tolerance (Ikehata *et al.*, 2008; Pfeifer *et al.*, 2005). Hence, when the types of mutations in response to UVR are seen in cells or explant skin (Cadet *et al.*, 2005; Courdavault *et al.*, 2004; Douki *et al.*, 1999; Douki *et al.*, 2003; Ravanat *et al.*, 2001) the prevalence of TT dimers is thought to be a consequence of the rapid repair of cytosine containing lesions. C to T transition mutations can be induced by both CPDs and 64PPs, and form the mutagenic basis of photocarcinogenesis (see section 1.4.1.3). It has been noted that rapid repair of 64PP in human skin *in vivo* correlates with the onset of erythema suggesting that its repair or the lesion itself may initiate the human erythema response (Young *et al.*, 1996). In mammalian cell mutagenesis studies however, 64PP are repaired quickly

(Mitchell and Nairn, 1989). Repair of Dewar isomers is as rapid as 64PP, but they do not induce C→T transitions in mammalian genetic studies *in vivo* (Lee *et al.*, 2000).

ROS induction occurs primarily in the UVA waveband with a peak at 390nm (Kvam and Tyrrell, 1997) thus the mutagenic potential of UVA was thought to be based on its induction of oxidative stress via $^1O_2$ (Zhang *et al.*, 1997b). 80% of 8oxodG formed can be attributed to $^1O_2$ (Cadet *et al.*, 2009), more commonly after UVA (Mouret *et al.*, 2006), and probably due to the transfer of UVA photons to endogenous photosensitisers (such as cytochromes, flavin, haem, NAD(P)H and porphyrins) (Cadet *et al.*, 2009).

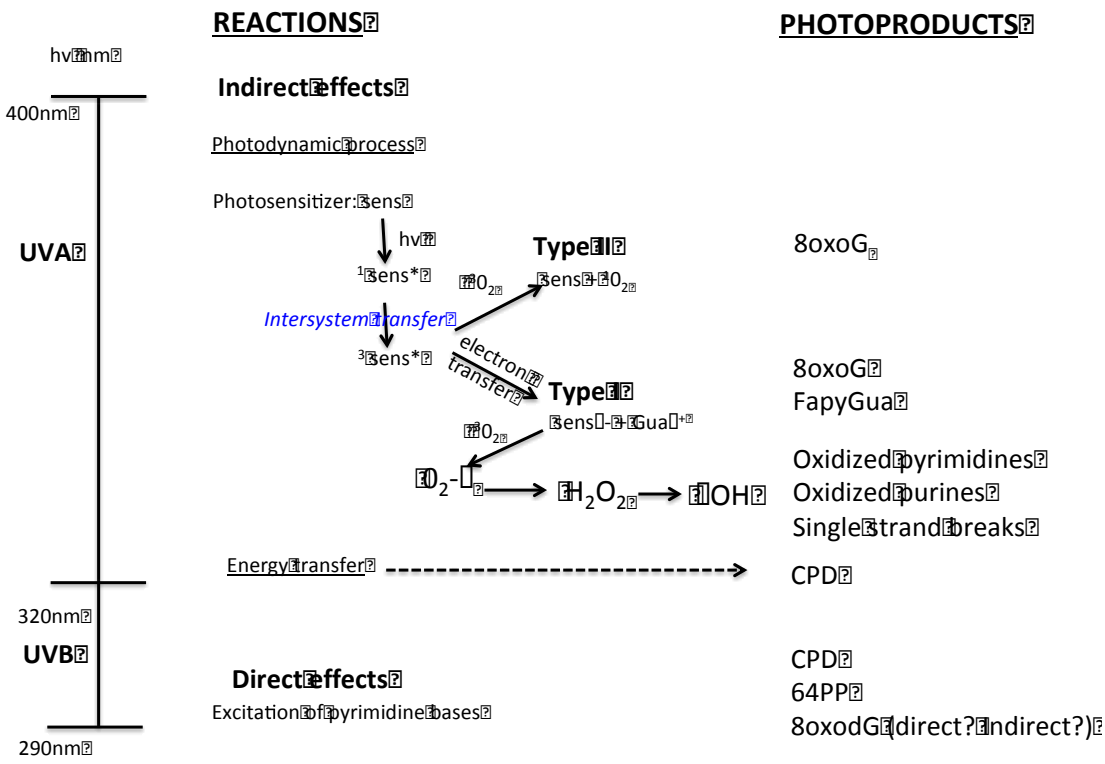


Figure 1.9 UVA and UVB mediated reactions to cellular DNA
Likely methods of DNA damage via UVA adapted from (Cadet *et al.*, 2009)

Early work showed that the most common base change after UVA exposure was the A:T→C:G (T→G) transversion (Drobetsky *et al.*, 1995). This was initially considered a UVA fingerprint mutation, due to 8oxodG which is found predominantly at the basal epidermis of precancerous solar keratoses and squamous cell carcinoma (SCC) (Agar *et al.*, 2004), also supported by other studies on human engineered skin (Huang *et al.*, 2009). ROS type mutations include T→G, G→T and T→A transversions (Halliday, 2005) due to a misincorporation of 8oxodG opposite adenine, *vice versa*, or oxidized thymine.

In vitro studies show that UVA readily induces C→T transitions that were previously widely associated with UVB induced CPDs (Kappes *et al.*, 2006; Runger and Kappes, 2008). The first evidence for CPD damage by UVA came from studies in *E.coli* using monochromatic 365nm (UVA1) (Tyrrell, 1973) although this was initially attributed to contamination by UVB (Hacham *et al.*, 1990). UVA CPDs are formed at TT dipyrimidine sites in UVA-irradiated human cells (Drobetsky *et al.*, 1995; Rochette *et al.*, 2003) and genotoxicity is probably the result of UVA1-induced cytosine-containing CPDs (Mouret *et al.*, 2006) that occur much less frequently. Transversion mutations are likely the minor contributor to UVA and UVB mutagenesis. This is because the UVA mutation spectrum in mammalian cells exhibits a predominance of the C:G→T:A mutation and in fact is the most common mutation produced by UVA and UVB (41% and 52% respectively) (Kappes *et al.*, 2006; Kappes and Runger, 2005). The G:C→T:A and A:T→C:G transversions occur at similar frequencies after UVA and UVB at 13% and 15% respectively) (Kappes *et al.*, 2006) suggesting minor roles in mutagenesis. In a more recent study, the C→T transition was found at a frequency of 65% and 85% for UVA and UVB

respectively (Ikehata *et al.*, 2008) with 8% mutations attributable to 8oxodG. UVA induced T→G mutations were also recovered at TT sites (Rochette *et al.*, 2003) or the same site as G:C→A:T transitions (Benjamin *et al.*, 2008) which might indicate that even this type of mutation was not always formed by an oxidized guanine, but by a CPD on the other strand. The exact role of 8oxodG in mutagenesis is unclear but mice that cannot repair 8oxodG (OGG1 8-oxoguanine DNA glycosylase-1) knockout show an increase in skin tumour development compared with wild type mice (Kunisada *et al.*, 2005) and G→T transversions, (G:C→T:A mutations) have been detected in the Brm gene in human basal cell carcinoma (BCC) and SCC (Moloney *et al.*, 2009) cell lines. Oxidative DNA damage has also been implicated in a wide variety of other pathological conditions, including cardiovascular disease, ageing, and neurodegenerative diseases (Cooke and Evans, 2007).

Mutation pattern	**Causative damage**	**Mechanism**
G:C→A:T transitions	C→T transition	CPD
G:C→T:A mutations	G→T transversion	8oxodG
A:T→C:G transversions	T→G transversion	8oxodG, oxidized thymine

Table 1.3 DNA damage mutations induced by UVA and UVB

It is now well established that the yield of TT CPD by UVA is larger than that of 8oxodG in both mammalian cells (Courdavault *et al.*, 2004; Douki *et al.*, 2003; Kielbassa *et al.*, 1997; Zhang *et al.*, 1997a) and human skin (Burren *et al.*, 1998; Freeman *et al.*, 1989; Mouret *et al.*, 2006; Young *et al.*, 1998b) and the term solar UVR mutations more accurately implies the C→T transition mutation produced by UVB and UVA which if unrepaired over time leads to skin cancer. Chapter 3

discusses CPD formation at biologically relevant doses of UVA1 in comparison to UVB and mechanism of formation *in vivo.*

DNA repair

In humans, the excision of damaged or inappropriate bases from the genome by multistep biochemical reactions are referred to nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR). The principle of NER involves sensing UVR induced photoproducts, splicing out the damaged region, inserting new bases to fill the gap, followed by ligation of the two ends of DNA. Our understanding of its importance primarily comes from studies in xeroderma pigmentosum (XP) (Cleaver, 1968) in which there are mutational defects in different NER proteins. Clinically, these are described as complementation groups XPA-G and the XP variant of NER depending on the defective complementation repair group. Failure to repair dipyrimidine lesions results in multiple skin cancers at an early age and an increased (1000-10000 fold) susceptibility to UVR induced skin cancer (Fassihi, 2013; Grampurohit *et al.*, 2011). NER consists of two sub-pathways: global genome repair (GGR) which repairs damage in both transcribed and untranscribed DNA strands in active and inactive genes throughout the genome and transcription coupled repair (TCR) where repair is selectively directed at the actively transcribing strand as a further preventative measure to reduce the chance of DNA damage being passed onto daughter strands during replication.

In GGR, damage is sensed by XPE and/or XPC whilst in TCR, RNA polymerase II stalls at a DNA lesion and cockayne syndrome complementation groups A and B (CSA and CSB) are recruited. Repair in both processes then continues through a

common transcription factor replicosome complex consisting of 30 distinct proteins (Friedberg, 2003; Sugasawa, 2010) (Figure 1.10) enabling DNA to unwind, DNA damage removal and then repair by DNA polymerase.

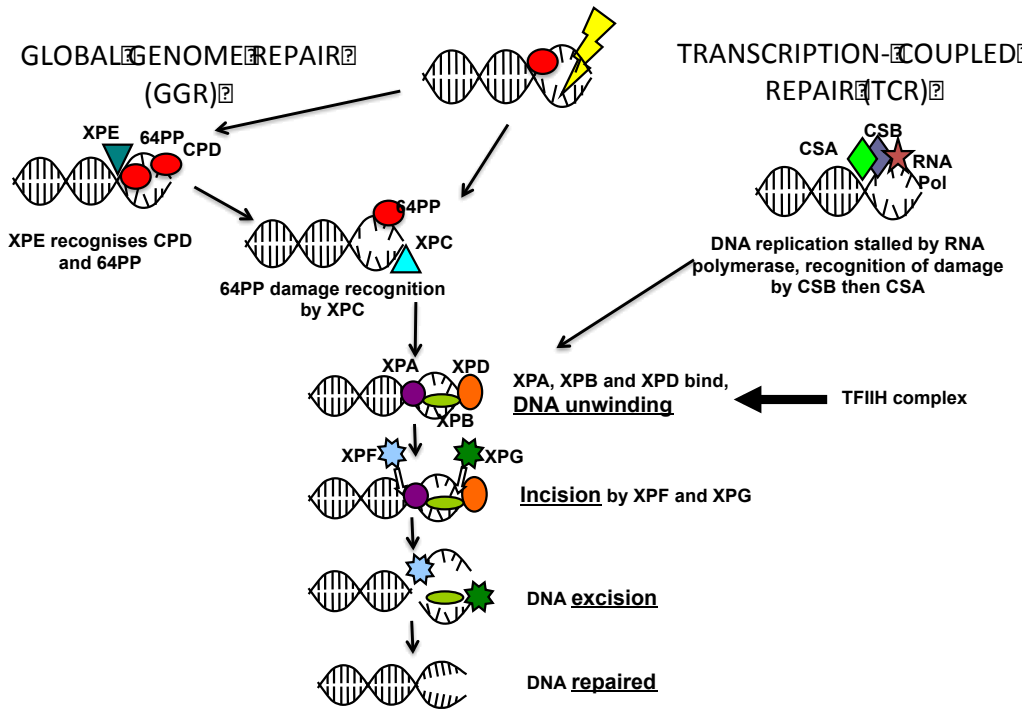


Figure 1.10 Repair of UVR induced DNA damage by NER
Adapted from (Sugasawa, 2010)
This shows that 6-4PP are recognized by both XPC and XPE whilst XPE must be in place first to recognize CPD before a complex can form with XPC. This could explain how 6-4PP might also be repaired more rapidly than CPD as they can independently be sensed by two complementation proteins (Mitchell and Nairn, 1989).

DNA repair is error prone and a separate 'overseer' of NER is a DNA damage tolerance process known as translesional synthesis (TLS). This is mediated by DNA polymerase η (Pol η) and effectively bypasses TT dimers, accurately placing A opposite (known as the A rule). This ensures an error free bypass of UVR induced lesions and those patients who are unable to participate in this, have a mutation in the Pol η gene (XP variant) and are extremely susceptible to skin cancers.

BER recognises damage to non-distorting single base modifications caused by oxidation: 8oxoGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG, FapyA), alkylation (methylation of bases producing 7-methlyguanine, 5-methylcytosine), hydrolysis or deamination (hypoxanthine from deaminated adenine or xanthine from deaminated guanine). BER is initiated by DNA glycosylases that cleaves the glycosylic bond between the target base and deoxyribose sugar. This leaves an apurinic /apyrimidic site or naked sugar phosphate backbone attached to the deoxyribose sugar, commonly referred to as an AP site (Krokan *et al.*, 1997)) which is also cytotoxic and needs to be removed by an AP endonuclease. The resulting single-strand break can then be processed by either short-patch (where a single nucleotide is replaced) or long-patch BER (where 2-10 new nucleotides are synthesized).

MMR corrects erroneous insertion, deletion and mis-incorporation of bases during DNA replication and is also strand specific.

Photoisomerisation of trans-UCA

trans-UCA (2E)-3-(1H-imidazol-4-yl)prop-2-enoic acid), a deamination product of histidine is present in high concentrations in epidermal *stratum corneum* and upon exposure to UVR (280-310 nm) (McLoone *et al.*, 2005), undergoes photoisomerisation to its cis-isomer. Photoisomerisation by the UVA2 (330nm) (Gibbs *et al.*, 1993) and UVA1 regions (Kammeyer *et al.*, 1995) has also been reported.

De Fabo and Noonan (De Fabo and Noonan, 1983) first reported that *trans*-UCA was important in systemic immunosuppression as removal of the *stratum corneum* in

mice by tape stripping, also prevented UVR-induced systemic suppression of the contact hypersensitivity (CHS) response. In fact a topical application of *cis*-UCA suppresses both induction and elicitation of CHS responses to contact allergens, including oxazolone, 2,4,6- trinitrochlorobenzene and dinitrofluorobenzene in mice (Hart *et al.*, 1997; Kurimoto and Streilein, 1992). An example of a delayed type hypersensitivity (DTH) model involves infection with herpes simplex virus: mice were infected subcutaneously with the virus, and 8-10 days later challenged intradermally in the ear with inactivated virus to produce an immune response. The epicutaneous or intradermal administration of cis-UCA before infection significantly suppressed the DTH response in a dose-dependent manner as measured by ear swelling (Ross *et al.*, 1986). These observations formed the basis for proposing than *trans*-UCA is a chromophore for UVR-induced immune suppression. Subsequent studies using hairless mice showed that topical application of *trans*-UCA increases both the number and malignancy of skin tumours induced by chronic exposure to UVR, suggesting that immunosuppression by cis-UCA can also promote photocarcinogenesis (Reeve *et al.*, 1989) by enabling CPD persistence and thus incorporation into the next cycle of DNA replication.

In humans, it has been reported that the relative production of *cis*-UCA following a single exposure to UVR was significantly higher in individuals with a past history of BCC or malignant melanoma (MM) compared with healthy individuals (De Fine Olivarius *et al.*, 1998) and recently, it has been shown that higher levels of *cis*-UCA were detected in SCC but not BCC biopsies compared with those from healthy skin (Decara *et al.*, 2008). Following UVR exposure, cis-UCA alters antigen presenting function of LCs and enhances prostaglandin E2 (PGE_2) formation which

subsequently can inhibit tumour necrosis factor α (TNFα) production in human peripheral blood mononuclear cells (PBMCs) (Hart *et al.*, 1993) and increase expression of IL10 mRNA (el-Ghorr and Norval, 1995; Holan *et al.*, 1998), resulting in immunosuppression and a lack of immunoregulated clearance of DNA photoproduct.

1.4.1.2 Cellular

Immunomodulation

See section 1.9 The immune response to UVR

1.4.1.3 Clinical

Inflammatory reactions including erythema

Erythema (or sunburn) is an acute inflammatory response of the skin to UVR and is associated with increased blood flow and sensitivity to thermal and mechanical stimuli, and a series of molecular/cellular changes (Harrison *et al.*, 2004; Rhodes and Lim, 2007). UVR damages many tissue compartments including membrane phospholipids, proteins and nucleic acids, which trigger a variety of proinflammatory responses. There is an influx of PGE_2 and nitric oxide (NO) (Rhodes *et al.*, 2001) after UVR exposure. At lower doses (1 MED), erythema is primarily mediated by NO whilst at higher doses both NO and PGE_2 have cumulative vasodilatory effects. UVR also directly causes a release of the proinflammatory cytokines TNFα, interleukin (IL)1, IL6, IL8 and IL12, and upregulates nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and transcription factor (TF) activator protein 1 (AP1) via cytokine signal

transduction pathways (mitogen activated protein kinase (MAPK): p38, JNK and ERK) that control expression of a wide range of genes (see Figure 1.14).

Vitamin D synthesis

Vitamin D is a group of fat soluble hormones comprising vitamin D_3 (cholecalciferol) and D_2 (ergocalciferol), responsible for enhanced intestinal absorption of important metal ions in the body including calcium, phosphate, magnesium, and zinc. Dietary sources equate to ~10% of vitamin D_3 and include oily fish (herring, salmon, sardines), liver and egg yolk. Vitamin D_2 is found as ergosterol in alfalfa, rye and mushrooms), as well as fortified foods in the US (milk, margarine and cereals). Both D_2 and D_3 are also available in vitamin supplements, however the major source of vitamin D is D_3, is formed following UVR absorption in the skin (λ_{max} at~297-300nm (Adams *et al.*, 1982) with no production above 315nm (Norval *et al.*, 2010)). The first step in cutaneous D_3 synthesis is absorption of UVB by 7dehydrocholesterol (7DHC) (provitamin D_3) (Webb *et al.*, 1988), in all layers but predominately the spinous and basal layers of the epidermis (Webb and Holick, 1988), and its resultant photoconversion to previtamin D (cholecalciferol). Concentrations of pre-vitamin D reach a maximum after a relatively low UVR exposure (<1MED) (Gilchrest, 2008), and further UVR exposure results in conversion of pre-vitamin to the inactive photoproducts lumisterol and tachysterol. Thus, this rate limiting step ensures that previtamin D levels remain at 10-20% of epidermal 7DHC concentrations (Adams *et al.*, 1982; Holick *et al.*, 1980), and vitamin D intoxication following UVR can not occur (Gilchrest, 2008). Previtamin

D_3 is then thermally converted to Vitamin D_3 which is released from the cell membrane into the extracellular space where it combines with a carrier protein (vitamin D binding protein) and is further metabolised to 25hydroxyvitamin D and finally 1,25dihydroxyvitamin D ($1,25(OH)_2D_3$) (calcitriol). These last two steps occur commonly in the liver and kidney respectively, although the whole pathway can take place in the skin (Lehmann, 2005) and other organs such as bone (Anderson and Atkins, 2008). $1,25(OH)_2D$ acts on vitamin D receptors (VDR) in tissue including colon, skin, breast and prostate to initiate a series of effects. The most established action of $1,25(OH)_2D_3$ is on the intestines and bone for calcium homeostasis. Recent work shows a lack of increased expression of VDR after multiple UVR exposures, which might also explain the absence of vitamin D and calcium toxicity in the body after UVR (Lesiak *et al.*, 2011). The binding of $1,25(OH)_2D_3$ to VDR results in proapoptotic and anticancer effects, modulating over 60 genes with prodifferentiating, antiproliferative, and antimetastatic effects on cells (Raimondi *et al.*, 2009). VDR is also expressed by BCC and SCC. Circulating $1,25(OH)_2D_3$ levels are maintained by a negative feedback mechanism that regulates renal 1α-hydroxylase and conversion of 25(OH)D to $1,25(OH)_2D_3$, thus plasma concentration of 25(OH)D is a good reflection of cumulative exposure to sunlight and dietary intake of vitamin D_3, and is widely regarded as a robust "gold standard" indicator of vitamin D status (Dawson-Hughes *et al.*, 2005).

It is hard to define optimal vitamin D status because different disease outcomes may depend on different levels. Table 1.4 reflects a general consensus view, but some authors advocate optimal levels of 75–100 nmol/L 25(OH)D (Bischoff-Ferrari *et al.*, 2006) or 80nmol/L (Heaney, 2000), but generally this is felt to be unachievable in

UK summer sunshine in white skin types (Rhodes *et al.*, 2010), as simulating summer UVR exposure produced sufficient vitamin D levels (50nmol/L) but not the proposed optimal status (80nmol/L). Levels below 50mmol/L are associated with a 30-50% increased risk of colon, prostate and breast cancer (Garland *et al.*, 2006). 25(OH)D <12.5nmol/L is generally accepted as severe vitamin D insufficiency and is associated not only with an increased risk of bone disease (Holick, 2007, 2008) but also of type I diabetes, hypertension and multiple sclerosis (Webb *et al.*, 2010). At Guy's and St Thomas' NHS Foundation Trust the optimum level has been set at ≥50nmol/L, as not only will this prevent bone disease but also this threshold level prevents compromise of macrophage/monocyte activation as part of the innate immune response (Holick, 2007). Active $1,25(OH)_2D_3$ can also reverse UVR-induced DNA damage and photoimmunosuppression in some models (Kuritzky *et al.*, 2008).

Vitamin D status	**Serum 25(OH)D level (nmol/L)**	**ng/mL**
Sufficiency	>50	>20
Insufficiency	25–50	10-20
Deficiency	12.5–25	5-10
Severe deficiency	<12.5	<5

Table 1.4 25(OH)D values in nmol/L and ng/mL
Targets used by the biochemistry laboratory at Guy's and St Thomas' NHS trust, and adapted from Pedersen *et al* (2008), where 1ng/mL = 2.5nmol/L. These units are used interchangeably in the literature.

Adequate bone health is thought to be achieved with supplementations of 800-1000IU of daily vitamin D_3 or 5000IU a month (Heaney, 2000; Lehmann, 2005) as a standard dose of 400IU (and 1000mg calcium) (1μg= 40IU) does not appear to significantly reduce hip fractures in a prospective study of 36,000 women (Jackson *et al.*, 2006). Fortified foods contain about 400IU per 8oz milk/juice/butter, salmon:

fresh (600-1000IU), farmed (100-250IU) per 3.5oz, sardines 300IU, mackerel 250IU, canned tuna 230IU, egg yolk 20IU, and one teaspoon of cod liver oil 400-1000IU. 200IU (5mcg) daily for 2-3months will increase vitamin D by 5nmol/L in comparison, 0.5MED UVB whilst wearing a swimsuit produces 10,000IU vitamin D (Holick, 2007)

UVR induced Vitamin D

People living near the equator who are exposed to sunlight without sun protection, have robust levels of 25(OH)D above 80nmol/L (Lee *et al.*, 2007). There has been work suggesting that people living at high latitudes are at increased risk of various cancers such as Hodgkin's lymphoma, colon, pancreatic, ovarian, breast compared to lower latitudes likely related to lower vitamin D levels (Garland *et al.*, 2006). Extrapolation of the CIE action spectrum for conversion of 7DHC to previtamin D (produced *ex vivo* on type III skin types) (Holick *et al.*, 1980; MacLaughlin *et al.*, 1982) shows that at mid-latitudes (around 40°) a fair skinned person should achieve maximal vitamin D_3 synthesis (after suberythemal exposures) within 5–10 minutes during midday on a summer day if this is done 2–3 times weekly. The time should be increased to 30 minutes for dark skin or if the sky is cloudy and exposure should to be on the face and forearms (Holick, 2007; MacLaughlin *et al.*, 1982). Few studies have actually measured sunlight induced conversion of 7DHC to previtamin D_3 at different latitudes at different times of the year. One group analysed this reaction using a solution of 7DHC exposed to sunlight for one hour on a cloudless day over the noon period (Webb *et al.*, 1988) and found no production of vitamin D_3 during the winter months at 42°N (Boston) or at 52°N (Edmonton), even if the exposure time was extended to 3h (Webb *et al.*, 1988). In the mid-summer months

of June and July, vitamin D_3 levels are produced at 52°N. We know that in the summer months in the UK, 80nmol/L levels are unachievable (Rhodes *et al.*, 2010), however if these levels can be produced by late summer (Webb *et al.*, 2010) this is associated with winter sufficiency (50 nmol/L) probably as excessive vitamin D formed in the summer is stored in fat for winter use. This group suggested that rather than a year round phenomenon, optimum vitamin D level should be considered as an end of summer status. Darker skin type individuals were unable to synthesise optimal vitamin D levels and a group of 15 Asian individuals exposed to solar simulated radiation (SSR) for 6 weeks to 35% body surface area would probably need oral supplementation (Farrar *et al.*, 2011) as they were unable to synthesize sufficient vitamin D over the summer months. This is likely to be related to melanin absorption attenuating UVB induced vitamin D synthesis. Recent work from our group (*unpublished*) show that in 50 individuals with skin types I/II and V/VI, the rate of Vitamin D synthesis surprisingly did not vary but baseline vitamin D level was race dependent with skin types V/VI having the lowest levels. This is supported by a recent audit on vitamin D levels in our photodermatology clinic where we also found lowest vitamin D levels in patients with skin types V/VI (21nmol/L) compared with skin types I/II (41nmol/L, $p<0.001$). A recent study suggests that sun seeking behaviour (sun and ski holidays) results is an increase in vitamin D although as there is concomitant DNA damage, thus vitamin D status is more safely mediated through oral vitamin D supplementation (Petersen *et al.*, 2014). Previous work suggesting that vitamin D can be influenced through sunbed use (Tangpricha *et al.*, 2004; Thieden *et al.*, 2008) or that sunscreen use should be minimized to get more vitamin D production (Youl *et al.*, 2009) is no longer

considered safe due to long term risks of photocarcinogenesis, although tanners have been shown to have robust levels of 25(OH)D (112nmol/L) and a higher bone density compared to non-tanners at the end of winter (Tangpricha *et al.*, 2004).

Sunscreens were initially felt to inhibit vitamin D photosynthesis (Matsuoka *et al.*, 1987) and by definition allow transmission of 1/SPF of incident erythemally weighted UVR (Gilchrest, 2008), i.e. $1/15^{th}$ or 7% for an SPF 15 product. Since users customarily apply half or less of the FDA/EC stipulated amount required to generate the stated level of protection ($2mg/cm^2$) (Bech-Thomsen and Wulf, 1992), they achieve far less protection, and calculations show that with an SPF 15 sunscreen, cutaneous vitamin D synthesis can occur after 10-20minutes of solar UVB exposure (Gilchrest, 2008). Supporting this, a recent *in vivo* study in Tenerife, Spain (*data unpublished)* showed that in skin type I/II individuals, judicious application of a broadband sunscreen will reduce UVR induced erythema however will not effect vitamin D synthesis. It is also important to note that exposure to 1 MED whilst wearing a bathing suit is equivalent to ingestion of approximately 20000IU of vitamin D_2. The greatest change is also seen in those individuals with the lowest baseline suggesting that the dose response relationships for vitamin D synthesis from UVB might be more complex.

Tanning

Three "tanning" processes occur in response to UVR: immediate pigment darkening (IPD), persistent pigment darkening (PPD) and melanogenesis or delayed tanning (DT) (Young, 2006).

IPD refers to the transient grey discoloration (Moan *et al.*, 2012b) of the skin due to immediate photooxidation of existing colourless melanin precursors following exposure to predominantly UVA wavelengths (established peak at 340nm) (Irwin *et al.*, 1993) and visible (400-500 nm) light (Pathak *et al.*, 1962), and fades within 15 minutes (Rhodes and Lim, 2007). This occurs at low doses (1-4J/cm^2 UVA) (Routaboul *et al.*, 1999) and the colour varies with individual complexion. It may be almost undetectable in fair-skinned individuals, but is easily observed in skin of types IV (or darker) (Young, 2006) due to the larger amounts of melanin (particularly eumelanin) in such skin (Brenner and Hearing, 2008) and is a reversible process.

IPD does not offer protection against erythema (Honigsmann, 2002) and its biological importance is unclear, although it has been recently suggested that its oxidized products absorb in the visible wavebands and thus prevents photodamage by visible light to other important molecules such as 5-Methyltetrahydrofolate (5MTHF) in the blood (Moan *et al.*, 2012b). 5MTHF deficiency may increase the risk of cardiovascular diseases, colorectal carcinoma and megaloblastic anaemia (Strickland *et al.*, 2013).

Development of a persistent brown colour (Miyamura *et al.*, 2007) in response to larger UVA doses (>10J/cm^2) in Caucasian skin peaking 2h post irradiation and lasting for up to 24h and sometimes 3-5 days later (Hwang *et al.*, 2011) is known as PPD and was first described in the 1970s (Kaidbey and Kligman, 1979). It is often perceived as a UVA tanning response (Rhodes and Lim, 2007) due to a permanent oxidation of melanin precursors (Wolber *et al.*, 2008). The action spectrum of PPD declines very slowly in the UVA region (Moyal *et al.*, 2000) and thus PPD been used

as the endpoint to assess UVA photoprotection by sunscreen (see 1.7.1 Sunscreens) to give a UVA protection factor (PF); the ratio of MPD (minimal pigmenting dose or PPD) with sunscreen compared to without sunscreen (Moyal *et al.*, 2000). Interestingly the IPD was recently suggested to be a more useful marker for UVA protection as it is less time consuming to produce, and needs lower UVA exposures (Hwang *et al.*, 2011), although it is a less stable response to UVA than PPD.

IPD and PPD proceeds differently in fair-skinned individuals than in darker persons (Monash, 1963). In fair skinned individuals, the MED is achieved before IPD (Lavker *et al.*, 1995), as there are very small amounts of melanin and its precursors, thus UVB is attenuated relatively less and so smaller doses produce erythema. Repeated exposures will result in cumulative erythema, and importantly a formation of a small amount of pigment, which means that although it is completely negative initially in some individuals, it may become slightly positive and a marker of previous UVR exposure. The process of tanning in dark skin pursues a different course. Here, the MED dose needed is higher than the dose to produce IPD and exposure to the sun produces considerable pigment darkening due to larger amounts of melanin precursors, with prolonged or repeated exposure increasing the duration of pigmentation to many months. Tanning is mostly due to IPD and PPD and sunburn is rare (Monash, 1963).

Delayed melanogenesis (DT), facultative pigmentation or neomelanogenesis is due to stimulation of new melanin synthesis by basal epidermal melanocytes which is then transported via dendrites to adjacent keratinocytes and redistributed towards the surface of the skin (Tadokoro *et al.*, 2005). The density of melanin varies with body site and declines with age (Whiteman *et al.*, 1999). Melanin is synthesised either as

dark-coloured brown–black insoluble eumelanin or light-coloured red–yellow, alkali soluble, sulphur-containing phaeomelanin. More eumelanin is produced in skin type III compared to skin type I, however no correlation has been observed for phaeomelanin (Thody *et al.*, 1991). Eumelanin is thought to be the major factor in the photoprotective properties of melanin, which when induced in white skin types, results in a protection factor of about 2–3 against DNA photodamage and erythema as well as a visible tan (Agar and Young, 2005). New melanin is eventually redistributed to the *stratum corneum* and is evident ~3 days post irradiation. The tan fades when the surface layer is shed over several weeks, although this can persist depending on complexion and UVR dose protocol (Sheehan *et al.*, 2002; Sheehan *et al.*, 1998; Young, 2006). There is *in vitro* evidence that products relating to phaoemelanin may have photosensitising properties contributing to the skin cancer susceptibility of people with red hair (Vincensi *et al.*, 1998) and recent mice studies show that the free radicals produced by UVA photosensitized phaeomelanin may increase the risk of melanoma (Noonan *et al.*, 2012).

The action spectrum for DT in human skin *in vivo* shows a peak at 290nm (Parrish *et al.*, 1982), similar to the peak for erythema (Parrish *et al.*, 1982) and CPD induction (Young *et al.*, 1998a) and is thought to occur predominantly following UVB (Moan *et al.*, 2012b; Wolber *et al.*, 2008). Larger doses of UVA will also evoke DT and studies show that the amount of UVA required to cause melanocyte activation and melanogenesis stimulation is more than 100 J/cm^2 in skin type III and IV (Beitner, 1986). This amount of UVA is over 5 times more than the dose at which UVA PPD occurs and is reported to correspond to more than 8h of sunlight in midsummer (Maeda and Hatao, 2004). Thus, the predominant response by UVA is not

melanogenesis. Recently, it was reported that although UVB, UVA and SSR showed comparable tanning responses, eumelanin and phaeomelanin levels were much higher only after UVB or SSR exposure (Wolber *et al.*, 2008) and not UVA. A recent gene study shows that the pigment changes after repetitive doses of UVA and UVB are entirely different (Choi *et al.*, 2010) supporting the concept that they occur via different mechanistic processes (see below on gene differences).

Pigmentation and melanocyte precursors

UVR induced DT is due to an increase in tyrosinase activity (seen 72h following UVB (Lavker and Kaidbey, 1982)) stimulating the rate limiting step: the conversion of tyrosine to L-DOPA. Other precursors include dopaquinone, 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). DHICA can be methylated to produce 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MICA) and 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MICA) in the cytoplasm of melanocytes (Maeda and Hatao, 2004) to eventually yield melanin.

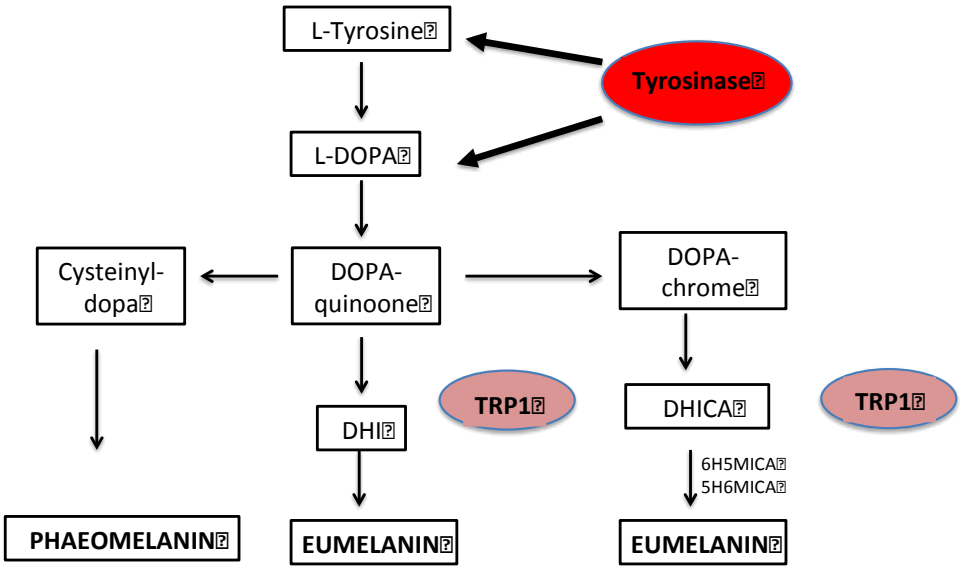


Figure 1.11 Biochemical events in melanogenesis

PPD and IPD are due to photooxidation of preexisting DHI, DHICA and 6H5MICA, which gives the characteristic brownish black persistent pigmentation, without concurrent reddening. These are oxygen driven processes as DHICA and 6H5MICA *in vitro* will only produce a colour change following the application of hydrogen peroxide (a prooxidant) (Maeda and Hatao, 2004). In fact repeated UVA exposure produces similar changes to IPD and PPD, which reflects oxidative changes in pre-existing melanin and its precursors (Miyamura *et al.*, 2007; Wolber *et al.*, 2008).

Mechanisms of neomelanogenesis

Tyrosinase activity and downstream melanin synthesis can be stimulated by UVR induced release of membrane-associated diacylglycerol (DAG) from keratinocyte plasma membranes, which activates PKC-β and in turn activates tyrosinase in melanocytes (Friedmann and Gilchrest, 1987). A small degree of tyrosinase stimulation also occurs through fibroblasts, neurons and mast cells, (Eller and Gilchrest, 2000). A recent study looking at gene expression after multiple UVR doses *in vivo* humans showed that UVB is a strong stimulator of various pigment related genes, such as tyrosinase (TYR) gene, tyrosinase-related protein 1 (TRP1) and dopachrome tautomerase (DCT), as well as the transcription factor microphthalmia-associated transcription factor (MITF) (Tadokoro *et al.*, 2005) and SSR is more effective in eliciting these effects than UVB alone suggesting a synergistic effect of UVA and UVB on melanogenesis (Choi *et al.*, 2010). UVA alone did not induce such upregulation of pigment cell-specific genes. Melanogenesis is also mediated via DNA photodamage (Gilchrest and Eller, 1999). *In vitro* experiments show that topical DNA repair enzyme (T4N5) treatment of murine S91 melanoma cells and human melanocytes exposed to SSR demonstrated

greater melanogenesis than when treated with heat-inactivated enzyme (Gilchrest *et al.*, 1993). The release of single stranded DNA fragments during CPD repair is thought to stimulate melanogenesis by increasing tyrosinase activity leading to an increase in tyrosinase protein and down stream new melanin formation (Gilchrest and Eller, 1999). This might explain how skin types V/VI who may have a faster DNA repair capacity release more DNA fragments stimulating increased tyrosinase activity and contributing to the deeper darkening of the skin (Barker *et al.*, 1995).

UVR induced DNA damage induces p53 (Chen *et al.*, 2014; Eller *et al.*, 1996) which is also a mediator of melanogenesis. p53 causes an upregulation of proopiomelanocortin (POMC), which is then processed to adrenocorticotrophic hormone (ACTH) and α-melanocyte stimulating hormone (α-MSH). Secreted αMSH binds to the melanocortin 1 receptor (MC1R) on melanocytes and via an increase in cAMP, increases tyrosinase activity.

1.4.2 Chronic effects of UVR on the skin

The hallmarks of chronic exposure to UVR are photocarcinogenesis and photoageing.

Photocarcinogenesis

It is common practice to use the term skin cancer for 3 main types of tumours derived from epidermal cells (Young and Wikonkal, 2007): MM from melanocytes, SCC and BCC from keratinocytes. MM is the 5th most common cancer in the UK, accounting for 4% of all new cases. Over the last 30 years, the incidence of MM has increased more than for any other common cancer in the UK (Parkin *et al.*, 2011) and in 2011, there were 13,348 new cases in the UK. SCC and BCC are collectively

known as non-melanoma skin cancer (NMSC) although a minority remainder comprises a mixed group of rare skin cancers including Merkel cell carcinoma, which has an extremely poor prognosis. NMSC are extremely common and in 2011, there were 102,628 cases registered in the UK: BCC (74%) and SCC (23%) although this might be an under representation as many cancer registries record only the first skin cancer of each histological type per person and often information on small NMSCs treated in primary care or the private sector never reach the registries (Brewster *et al.*, 2007; de Vries *et al.*, 2012).

The concept that chronic sun exposure might cause NMSC was first introduced in 1894 (Unna, 1894) with the observation that sailors, who were chronically exposed to large amounts of sunlight, were predisposed to skin cancers on sun exposed sites. Overall cumulative UVR dose is a good predictor of SCC risk, particularly in lighter skin types where skin cancer incidence is higher compared to darkly pigmented individuals (Leiter and Garbe, 2008). Interestingly, this may not be the case with vitiligo, a disease in which there are isolated depigmented patches of skin. Recent work found a significant decrease in the incidence of MM, BCC and SCC particularly in affected areas compared to normal skin, and after multiple courses of UVB phototherapy (Teulings *et al.*, 2013). The link between BCC and sun exposure was unclear until a cardinal study demonstrated that mice with mutant PTCH and chronically exposed to UVR produced BCC (Aszterbaum *et al.*, 1999). In contrast to SCC, the incidence of BCC (Kricker *et al.*, 1995) and MM (Newton-Bishop *et al.*, 2011) are related to intermittent large dose solar UVR exposure in childhood and adolescence. MM are found typically on the legs of women and backs of men, areas that are typically covered up but receive intermittent UVR exposures (Godar *et al.*,

2009). Studies show that regular recreational sun exposure is associated with photoadaptation and vitamin D synthesis, which are thought to be protective whilst intermittent acute sunburn exposure causes more damaging effects, furthermore, outdoor workers, chronically exposed to sunshine, do not get MM (Davis *et al.*, 2002; Godar *et al.*, 2009; Newton-Bishop *et al.*, 2011). The proportion of UVA in sunlight (UVA/UVB) increases with increasing latitude, as does the ratio of MM/SCC. This suggests that the greater proportions of UVA in sunlight as found in temperate latitudes, may play a role in MM induction (Moan *et al.*, 2012a) although a lack of vitamin D production at temperate lattitudes must also contribute (Godar *et al.*, 2009). MM likely occurs through two process: an initiation stage or acute sunburn by UVB, and a propagation stage, possibly by UVA which can be transmitted through clothing (Mitchell and Fernandez, 2012) with two different wavelength specific pathways of melanoma induction (Noonan *et al.*, 2012)

In terms of the relative contributions of UVR on carcinogenesis, studies with polychromatic sources in hairless albino mice have been used to generate an action spectrum for non-melanoma skin cancer (known as SCUP- Skin Cancer Utrecht-Philadelphia) (de Gruijl *et al.*, 1993) which has been mathematically corrected for differences in UVR transmission between murine and human epidermis to produce an action spectrum for SCC induction in humans (de Gruijl and Van der Leun, 1994). This spectrum when considered with the action spectrum of CPD (Freeman *et al.*, 1989) clearly indicates the importance of UVB induced CPD in the formation of SCC as the action spectra are similar to ~330nm (see Figure 1.12). From early on CPD was known to exhibit biological activity (Setlow and Setlow, 1962), and recently it has been shown that DNA is a chromophore for erythema up to 340nm

(Young *et al.*, 1998a). There is a second SCC induction peak around 380nm (within the UVA1 spectrum), of which the second erythemal peak (~360nm) may play a contributory role in its formation (Anders *et al.*, 1995). The action spectrum for melanoma induction in *Xiphophorus* fish is different from the SCC action spectrum as there is a greater contribution from UVA (Setlow *et al.*, 1993). If the Setlow study is weighted for the relative presence of UVA in sunlight, 90-95% of human MM induction can be primarily attributed to UVA wavelengths. although this has been recently refuted (Mitchell *et al.*, 2010). The increased sensitivity to UVA can be seen when comparing melanoma induction to UVB (300nm) where UVA1 (360nm) is only one order of magnitude less potent. This is not the case with SCC induction where UVA1 is 3 orders less potent at induction then UVB.

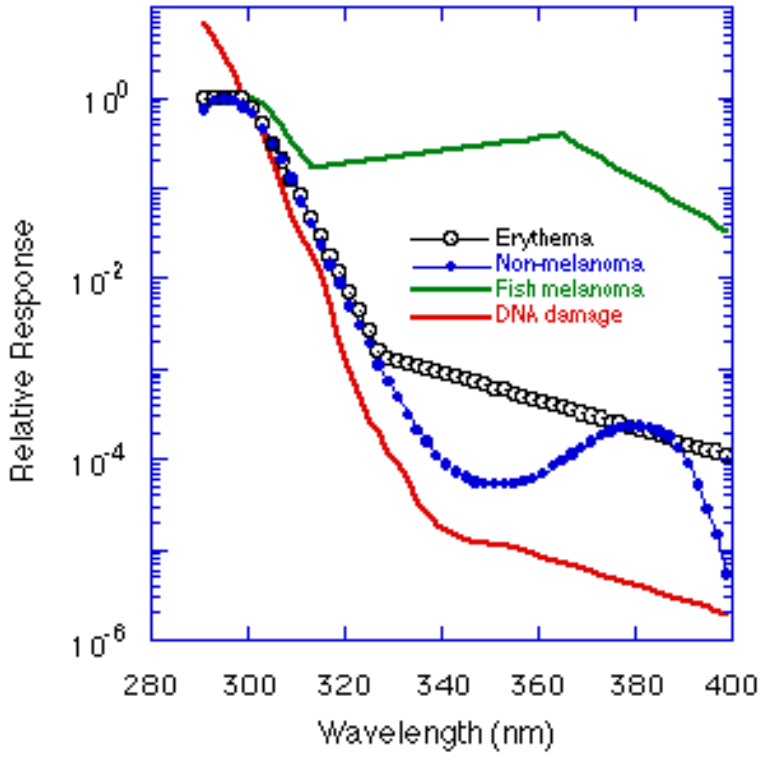


Figure 1.12 Action spectra for selected UVR endpoints
Adapted from (CIE, 1998) for erythema, (de Gruijl *et al.*, 1994) for SCC, (Setlow *et al.*, 1993) for melanoma, (Young *et al.*, 1998) for DNA damage.

Constitutive skin pigmentation protects against UVR-induced damage from a single MED exposure (Del Bino *et al.*, 2006; Tadokoro *et al.*, 2003) and there is more CPD damage in skin types I/II compared to II/IV after approximately 2-3SED (Mouret *et al.*, 2011b). Melanin has a major role in individual skin UVR sensitivity, and there is an inverse relationship for the risk of skin cancer (Miyamura *et al.*, 2007) with a 70-fold higher risk factor for BCC and SCC in Caucasian Americans compared with black or African-American skin (Lea *et al.*, 2007) and an 18-fold (women) and 26-fold (men) lower incidence of melanoma in black compared to white individuals (Horner *et al.*, 2009).

The stages of photocarcinogenesis are initiation and promotion. DNA damage causes inactivating mutations in selected tumor suppressor genes (TSG), or activating mutations in oncogenes. During the promotion stage, mutant keratinocytes undergo additional biochemical changes, and clonal expansion, that may result in visible pre-malignant papillomas. Some of these will acquire a further mutational alteration that allows them to become invasive carcinomas. Probably the most extensively studied TSG is TP53 whose crucial role is the orchestration of a cell's response to UVR-induced DNA via its activation through signal cascade induced phosphorylation and subsequent triggering of DNA repair enzymes, cell cycle arrest, DNA synthesis and programmed cell death: apoptosis (sunburn cells (SBC)) (Harris and Levine, 2005), all of which suppress photocarcinogenesis (see Chapter 4). The C→T transitions and CC→TT resulting from replacement of cytosine have been known for many years to be UVR specific. In one study, over 90% of SCC were shown to have a mutation in the TP53 gene and approximately

60% were found to be C→T or tandem CC→TT transitions (Brash *et al.*, 1991). These signature mutations were also found to occur in 90% of precancerous actinic keratosis (Ziegler *et al.*, 1994). In BCC, mutational analyses of TP53 show that 60% of tumours harbour the UVR signature transition (Ziegler *et al.*, 1993), which is also found in the PTCH gene (Zhang *et al.*, 2001).

UVR exposure is a known environmental risk factor in the development of MM (Leiter and Garbe, 2008; Pfeifer *et al.*, 2005) however, only recently a link to the characteristic UVR mutation has been established. Advances in sequencing technology make it possible to comprehensively catalogue a melanoma cell line where the most common somatic mutation karyotyped was the C→T transition in 90% of base substitutions throughout the genome and the CC→TT in 70% of dinucleotide substitutions. Although a link with p53 and UVR has not been established in MM (Ragnarsson-Olding *et al.*, 2002; Zerp *et al.*, 1999), its transcriptional activity is thought to be inactivated (Houben *et al.*, 2011) in MM.

An additional biochemical change that occurs during the promotion and progression stages of UVR carcinogenesis is increased expression of cyclooxygenase-2 (COX2) which converts arachidonic acid to PGE_2, and whose upregulation can be easily detected in actinic keratosis (AK), SCC and BCC. Prostaglandins have been associated with a broad range of functions including inflammation, angiogenesis and immunosuppression. COX2 inhibitors such as diclofenac are successfully used to treat AKs (An *et al.*, 2002) and prevent UVR induced skin cancers in animal models (Fischer *et al.*, 1999). Interestingly, individual who regularly take NSAID have a lower incidence of cutaneous SCC than those who do not, although a causal

relationship has not established (Butler *et al.*, 2005).

Photoageing

Skin ageing occurs as two distinct phenomena: intrinsic chronological ageing which are the changes attributable to the passage of time and can be accelerated by external agents such as cigarette smoke (Lahmann *et al.*, 2001; Morita *et al.*, 2009) and lifestyle factors such as stress, illness and pollution (Flament *et al.*, 2013), and photoageing: the superposition of changes caused by chronic UVR exposure (Yaar, 2007b). Intrinsic ageing is regarded by many authorities as a cancer prevention mechanism (Campisi, 1996) as the eventual arrest of cell cycle division known as 'replicative senescence' is thought to prevent proliferation of abnormal DNA and possible tumour induction. Intrinsic ageing of the skin presents as finely wrinkled, lax, dry, rough skin and a variety of benign neoplasms, whilst photoaged skin by definition affects predominantly the face (responsible for 80% of facial skin ageing (Flament *et al.*, 2013),) dorsal hands, forearms and upper chest. Two types are described in the literature (Wlaschek *et al.*, 2001): a severe atrophic subtype, with multiple telangiectasia, minimal wrinkle formation and a variety of premalignant lesions such as AK, and the Milian's citrine skin which presents with deep wrinkles, a leathery appearance, laxity, increased fragility, blister formation and impaired wound healing. White patients presenting with BCC are less wrinkled than peers of similar complexion and degree of photodamage (Brooke *et al.*, 2001) suggesting a possible protective role of wrinkling in the second subtype of photoageing.

Photoageing in darker skin types appears as dry, sallow (Yaar, 2007a) and wrinkling at later stages but also increasingly pigmented skin due to an increase in melanocyte

number and a consequent increase in melanin (Gilchrest *et al.*, 1979). In terms of wrinkling, generally fairer skin types are more severely affected than darker skin types, although african skin has been shown to have a higher baseline level of collagen I, III and fibrillin rich microfibrils (Chung, 2003) and these differences could related to UVR penetration properties in different skin types as demonstrated in skin cultures (Kaidbey *et al.*, 1979) in which black skin allows approximately 6% of UVB to be transmitted into the dermis compared with almost 30% into the dermis of white skin. Furthermore, only 18% of UVA is transmitted into black dermis compared with more than 55% penetration in white skin. The incidence of skin cancer is much lower in this phototype (Yaar and Gilchrest, 2007) see section 1.4.2 Photocarcinogenesis.

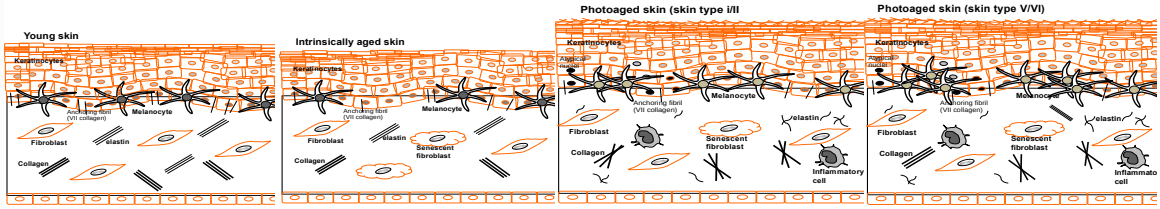
Histology of ageing

The dermis consist of collagens, (fibrillar: I, II, III, V, XI, anchoring: VI and VII) and elastic fibres encased in the dermal matrix (Langton *et al.*, 2010, 2012; Thurstan *et al.*, 2012) consisting of a gelatinous amorphous mix of glycoproteins such as fibronectin which stabilise fibres and give flexibility to the skin and proteoglycans (which have a lower protein content than glycoproteins) such as glycosamine which are mostly produced by fibroblasts and are rich in hyaluronic acids. These are associated with moisture retention. The different collagens in the skin exert different functions in different parts of the dermis: for example the anchoring collagens IV and VII are localised at the dermal epidermal junction (DEJ) (in the papillary dermis) where they play key roles in binding and stabilisation of tissue layers whilst the widely distributed fibrillar collagens I and III, found predominantly in the reticular dermis, form covalently bonded fibrils which resist tensile forces. Except

for collagen IV (Watson *et al.*, 2001a) these are all reduced in photoageing (Wlaschek *et al.*, 2001). The elastic fibres, which drive passive recoil and give the skin elasticity (Uitto, 1979), are composed of an elastin core and a microfibril scaffold (with a 10-20nm diameter), which in an adult is composed largely of fibrillin I (Watson *et al.*, 1999). The elastic fibres are sometimes referred to as tropoelastin (rich in valine and alanine as well as hydroxyproline). The key characteristic histological change in photoaged skin is a significant accumulation of dystrophic cross-linked elastic fibres sheathed in fibrillin rich microfibrils in the reticular dermis which gives it the amorphous appearance on histology and is classically termed solar elastosis (Bernstein *et al.*, 1994; Calderone and Fenske, 1995; Mitchell, 1967). Recent work has shown a significant decrease in fibrillin 1 protein in early photoaged skin (Watson *et al.*, 1999). Furthermore, an increase in mast cells, mononuclear cells and neutrophils have been reported in murine photoaged skin (Kligman and Murphy, 1996) likely mediated through an upregulation of IL1B (Qin *et al.*, 2014). The impact of UVA1 and UVB on solar elastosis is examined in Chapter 5.

With intrinsic ageing, the skin exhibits epidermal and dermal atrophy and a reduced number of mast cells and dysfunctional fibroblasts (which produce less collagen and more matrix metalloproteinases (MMP) see below). This can be explained by the age associated increase in senescent fibroblasts that become increasingly collagenolytic (West *et al.*, 1989)

A diagram comparing changes in the skin due to intrinsic and photoageing is shown below.



-Well balanced distribution of distinct cell types

-Clinical-smooth pale atrophic, dry
-fine wrinkles
-decreased epidermal/ dermal thickness
-general atrophy of ECM
-flattened DEJ
-decreased elastin
-decreased collagen (amount and thickness) and decreased fibroblasts (senescence)
-hypocellular

-Clinical-leathery, coarsely wrinkled (with deep furrows, yellowish skin, sallow
-acanthosis, mild hyperkeratosis (dryness)
-hypo/hypermelanosis
-loss of mature dermal collagen
-increased procollagen mRNA
-Loss of anchoring collagen fibres
-Decrease collagen I and III
-Dystrophic elastotic (tropoelastin) material in deep dermis
-decrease in microfribrils
-increase in mast cell, mononuclear, neutrophils (helodermatitis)

-Clinical-hyper-pigmented tanned skin
-more melanocytes and melanin in suprabasal layers
-reduced degree of wrinkling compared to white skin
-melasma more common

Figure 1.13 Histological changes in ageing and photoageing

MMP and photoageing

Extracellular matrix proteins known as MMP are a large family of proteases that share common structural and functional elements, and all contain a conserved zinc containing binding site that is found in their catalytic domain (Klein and Bischoff, 2011). MMP are released as proenzymes where a blocking cysteine residue must be removed in order to activate enzyme activity. This is largely in order to ensure MMP activity is focussed at target sites. Their main physiological function is ascribed to the modulation and regulation of extracellular matrix (ECM) turnover by direct proteolytic degradation of dermal ECM proteins and non-matrix proteins (Woessner, 1991) as well as liberation of biologically active cytokine growth factors and chemokine from their membrane anchored preforms. MMP upregulation forms part of normal physiological processes such as wound healing and angiogenesis (Chakraborti *et al.*, 2003).

There are many different MMP classification systems. The most biologically relevant system involves classification according to function, and subdivides MMP into collagenases (capable of degrading the triple helical collagens e.g. MMP1, MMP8, MMP10, MMP13), gelatinases which primarily act on type IV collagen and gelatin and include MMP2, MMP9, the stromelysins which cleave extracellular matrix proteins but not the triple helix of collagen and include MMP3, MMP10, MMP11 and the membrane type (MT) MMP (MMP14, MMP15, MMP16, MMP17, MMP24, MMP25), although it is quite clear that there are a series of MMP that do not fit into these groups, namely MMP7 and MMP12. There is also considerable overlap in function as MMP2 also has collagenolytic activity, as does MMP14.

Enzyme	MMP	ECM substrate
Collagenases		
Collagenase-1 or interstitial collagenase	MMP1	Collagens (**I**, II, VII, VIII, X) gelatin, aggrecan, veriscan, tenacin, entactin
Collagenase 2 or neutrophil collagenase	MMP8	Collagens (**I**, II, III, V, VII, VIII and X) gelatin, aggrecan
Collagenase 3	MMP13	Collagens (**I**, II, III, **IV**, IX, X, XIV) gelatin, aggrecan, fibronectin, osteonectin
Collagenase 4	MMP18	-
Gelatinases		
Gelatinase A or 72kDa gelatinase	MMP2	Collagens (**I**, **IV**, V, VII, X, XI and XIV), gelatin, **elastin**, fibronectin, lamnin-1, laminin 5, aggrecan
Gelatinase B or 92kDa gelatinase	MMP9	Collagens (**IV**, V, VII, X and XIV) gelatin, **elastin** aggrecans, fibronectin, entactin, osteonectin
Stromelysins		
Stromelysin1	MMP3	Collagens (III, **IV**, V, IX) gelatin, aggrecan, versican, perlecan, decorin, fibronectin, laminin, entactin, osteonectin
Stromelysin 2	MMP10	Collagens (III, **IV,** V), gelatin, casein, aggrecan,
Stromelysin 3	MMP11	Casein, laminin, fibronectin, gelatin, collagen **IV**
Matrilysin, enamelysin, metalloelastase		
Matrilysin	MMP7	Collagen (IV, X), gelatin, aggrecan, decorin, fibronectin, laminin, **elastin**, casein, transferrin
Matrilysin 2	MMP26	-
Enamelysin	MMP20	Amelogenin

Metalloelastase	MMP12	Collagen IV, gelatin, elastin, casein, laminin, fibronectin, enactin
Membrane type MMPs		
MT1-MMP	MMP14	Collagens (I, II, III), casein, elastin, fibronectin, gelatin, laminin, vitronectin, entactin, proteoglycans
MT2-MMP	MMP15	Fibronectin, laminin, entactin, aggrecan, perlecan
MT3-MMP	MMP16	Collagen III, gelatin, casein, fibronectin
MT4-MMP	MMP17	-
MT5-MMP	MMP24	-
MT6-MMP	MMP25	-
Other		
-	MMP19	Gelatin
-	MMP23	-
-	MMP21	-
-	MMP22	-

Table 1.5 Classification of MMPs
This table shows the substrates acted on by all MMP. These have formed the basis of enzyme activity staining protocols (*in situ zymography*) in an attempt to more accurately localise MMP in the skin (Mook *et al.*, 2003; Yan and Blomme, 2003)

MMP activity also occurs in pathological tissue destruction processes as found in chronic wounds, dermal photoageing, bullous skin disease, cancer invasion (MMP1 and MMP10 in SCC (Ramos *et al.*, 2004)) and MMP12 induction in epithelial SCC and BCC and head and neck SCC (Kerkela *et al.*, 2000), and metastasis (MMP12) and vulval cancer invasion (Kerkela *et al.*, 2002) and this has been extensively reviewed elsewhere (Deryugina and Quigley, 2006).

Pathological increases in MMP: MMP1, MMP3 and MMP9 form the basis of the photoageing process in human skin (Fisher *et al.*, 1996; Fisher *et al.*, 1997; Quan *et al.*, 2013) predominantly through AP1 induction. UVR induction of MAPK (JNK, ERK, p38) signalling activation causes an upregulation of AP1 (cFos-cJun) complex through cJun upregulation (Fisher and Voorhees, 1998) which occurs at 2h post irradiation and 8h maximally, without a concomitant increase in tissue inhibitors of matrix metalloproteinases (TIMP 1-4) (Quan *et al.*, 2009). Interestingly MMP2

upregulation is not mediated by AP1 (Fisher *et al.*, 1996). Upregulated MMP1 protein degrades collagen I and III and these accumulated collagen fragments also have an effect of further inhibiting fibroblast ability to produce new collagen. The net result is a degradation of collagen in the dermis, which reduces the structural integrity of the skin. UVR also causes transforming growth factor β1 (TGFβ1) suppression which decreases collagen I and III levels, whilst UVR activation of NFkB (which controls expression of a large array of genes involved in immune function and cell survival) directly upregulates MMP8 that is released from activated neutrophils and contributes to the dermal matrix remodelling process. Other UVR upregulated MMP include MMP10 in a SCC cell line (Ramos *et al.*, 2004) and MMP2 (Brenneisen *et al.*, 2002) and recently other work has shown that MMP11, MMP17, and MMP27 mRNA are upregulated in photoaged skin (Quan *et al.*, 2013). Work has extensively studied the mechanisms by which UVR induces MMP, and the wavelengths responsible for MMP1 and MMP3 induction have been shown to be distinct from the absorption spectrum of DNA (Brenneisen *et al.*, 1996) suggesting another mechanism of induction other than direct DNA damage. MMP can be triggered by CPD formation (Dong *et al.*, 2008) as well as indirectly via the generation of ROS and ROS induced lipid peroxidation (Brenneisen *et al.*, 1997; Brenneisen *et al.*, 1998; Scharffetter-Kochanek *et al.*, 1993). MMP may also be induced by mediators such as IL6/IL1 (Wlaschek *et al.*, 1993; Wlaschek *et al.*, 1994) and TNFα (Steenport *et al.*, 2009), which is readily induced in the skin by UVR, probably via the induction of CPD (Walker and Young, 2007).

Other mechanisms of photoageing

There is an emerging view that photoageing is the result of a repeated microscopic abnormal wound healing responses which coalesces into 'solar scars' (Fisher *et al.*, 1996) over time, although the free radial theory of photoageing due to repetitive protein oxidation in the dermis has also been entertained (Sander *et al.*, 2002; Yaar and Gilchrest, 2007), and supporting this is the lower constitutive levels of the antioxidant enzymes catalase and superoxide dismutase 1 and 2 (SOD1 and SOD2) in the dermis compared to the epidermis.

Genetic damage and instability outside the nuclear genome has been suggested to contribute to ageing. Cellular mitochondria generate energy (ATP) via a series of redox reactions mediated through the electron transport chain and antioxidants (NADH, FADH2, coenzyme Q). Despite these constitutive enzymes and mitochondrial BER, mitochondria DNA (mtDNA) is particularly sensitive to ROS induced damage and the mutation incidence for mtDNA is about 20 fold increased compared to nuclear DNA (Wlaschek *et al.*, 2001). Photodamaged skin has a higher mtDNA mutation frequency when compared with sun protected skin (Berneburg *et al.*, 2000) which also correlates with higher MMP1 levels.

UVA and UVB contribution to photoageing

Although the action spectrum for photoageing in humans has not been determined, the relative contributions of different wavebands in mice have been studied (Bissett *et al.*, 1989; Kligman and Sayre, 1991; Wulf *et al.*, 1989) and UVB is more potent than UVA. Because of its >10 fold greater abundance in terrestrial sunlight, far greater year round and day long average irradiance and greater average depth of

penetration into the dermis compared with UVB, UVA is still thought to play a large role in photoageing. There have also been reports of facial photoageing asymmetry in car drivers with more damage on the side of the face closest to the car window (Singer *et al.*, 1994) which is possibly more likely caused by UVA, and not UVB, penetrating through glass. Studies in reconstructed human fibroblasts *in vitro* showed that UVB primarily affected epidermal cells forming CPD and SBC whereas UVA induced apoptosis of fibroblasts located in the upper dermal compartment as well as secretion of MMP (Bernerd and Asselineau, 1998; Herrmann *et al.*, 1993). Histological examination of the skin after multiple sub-erythemogenic doses of UVA1, UVA2 and SSR showed that inflammatory infiltrates (lymphocytes, histiocytes and mast cells) appeared to be a predominantly UVA mediated dermal response (Lavker *et al.*, 1995).

Measuring photoageing

Several systematic approaches have been developed to measure photoaging. A photonumeric scale in which clinical parameters (fine wrinkling, coarse wrinkling, mottled pigmentation and sallowness) are graded from 0-8 showed 80% agreement by 7 independent dermatologists (Griffiths *et al.*, 1992) has also been adapted in white females (Larnier *et al.*, 1994), and a similar approach has been set up for Korean skin (Chung, 2003). Other approaches include assessing skin topography with a modified dermatoscope (Dermascore), using a corneometer to assess *stratum corneum* hydration and confocal microscopy visualisation of the various layers of the skin (Yaar, 2007b) and mechanical models have been developed to measure elasticity of the dermis (Pailler-Mattei *et al.*, 2008). These methods can be used as endpoint measurements and recently, the use of silicone moulds to obtain skin

surface markings has been used in over 900 adults on the backs of hands demonstrating the effectiveness of daily sunscreen use in retarding photoageing compared to ad hoc sunscreen users over a period of 4 years (Hughes *et al.*, 2013).

Treatment of photoaged skin

The gold standard pharmaceutical therapy for photoageing is the use of all trans retinoic acid (tRA). This is a derivative of vitamin A, and was first used to treat AK and acne in the 1970s (Pedace and Stoughton, 1971) and incidentally found to also improve periorbital wrinkles. Subsequent studies have shown that topical tRA improves fine wrinkling of the skin after 3-4 months of therapy with an increase in mean epidermal thickness (Kligman *et al.*, 1984) and similar efficacy can be achieved at 0.025% and 0.1% but with lower irritancy (tretinoin associated dermatitis) (Griffiths *et al.*, 1995). The mechanism of action has been extensively studied and shown to involve fibroblast stimulation and new collagen synthesis, via nuclear retinoid receptors (RAR and RXR) which prevents c-Jun activation, and hence AP1 expression and downstream reduction of MMP activation in the epidermis and dermis (Fisher *et al.*, 1996; Fisher *et al.*, 1998). Pretreatment with all trans retinoic acid also results in significant inhibition of UVR induced MMP1, MMP3 and MMP9 mRNA and protein levels with no effect on TIMP1 expression (Fisher *et al.*, 1997) although photosensitivity has been described (Birner and Meyer, 2001). There is also a partial restoration of fibrillin 1 that occurs after daily application of tRA measured over a 4 years (Watson *et al.*, 2001b).

1.5 Infrared and visible light

In direct sunlight, the temperature of human skin can rise above 40°C after the conversion of absorbed IR into heat (Cho *et al.*, 2009), and erythema *ab igne* characterised by reticular pigmentation, and histologically by the presence of solar elastosis in the dermis, is a well known heat induced skin condition (Kligman, 1982). Gene studies in human fibroblasts indicate that IR is a potent regulator of key signalling molecules such as ERK1/2, p38, JNK, PI3K/AKT, STAT3 (Calles *et al.*, 2010) and at physiological doses, corresponding to 2-3h of summer sunshine in central Europe (360-720J/cm^2), there is approximately 8 fold upregulation of MMP1 mRNA compared to control (Schroeder *et al.*, 2007). Mechanism of induction is thought to be absorption by copper in complex IV of the mitochondrial respiratory chain and generation of intramitochondrial ROS resulting in an activation of MAPK in cell cytoplasm and subsequent intranuclear transcription of MMP1 presumably via AP1 (Schroeder *et al.*, 2008). Multiple doses of IR on human skin *in vivo* over a period of 4 weeks increased MMP1 mRNA and protein levels and decreased procollagen 1 levels (Kim *et al.*, 2006) although chronic heat exposure in hairless mice causes an increase in MMP1 MMP3 and MMP12 (Chen *et al.*, 2005) but not MMP2, as well as clinically observed skin wrinkling (Kim *et al.*, 2005).

Comparing effects of the different components of sunlight in human volunteers after a 3h summer sunshine exposure in Korea, there appears to be a synergistic effect of IR and visible light in inducing slightly higher MMP1, MMP9 expression as well as a decrease in procollagen 1 levels (Cho *et al.*, 2008). The effects of IR on CPD induction has not been studied however visible light does not induce TT dimers.

Clearly IR contributes to photoageing however it will not be discussed further in this thesis.

1.6 Genetic effects of UVR on the skin

Following UVR absorption there is direct damage, as well as indirect damage to DNA, lipids, proteins in the skin via production of ROS. These can oxidize free lipids in the *stratum corneum* or keratinocyte membranes causing release of ceramides which via protein kinase mediates signal transduction (MAPK activation), activates AP1 resulting in expression of many stress response genes, including intercellular adhesion molecule 1 (ICAM1) on keratinocytes and vascular endothelial growth factor (VEGF), ICAM1 and E-selectin on endothelial cells which enables chemotaxis of neutrophils and lymphocytes (Hawk *et al.*, 1988) respectively to propagate the inflammatory response. AP1 also interferes with TGFβ transcription that ultimately blocks dermal collagen I and III production and also activates MMP (see Photoageing). Oxidized lipids are cleaved by phospholipase A_2 (PLA_2) to form arachidonic acid that is the substrate for COX1 and inducible form 2 (COX2). These enzymes convert arachidonic acid into prostaglandins and mediate many of the inflammatory reactions in the skin in response to UVR. MAPK activation (via c-Jun N-terminal kinases (JNK) and p38 signalling) can also occur via growth factor and growth factor receptor (epidermal growth factor receptor (EGFR). ROS induced protein oxidation leads to activation of damage response pathways leading to MMP production, inflammation and clinical signs of erythema.

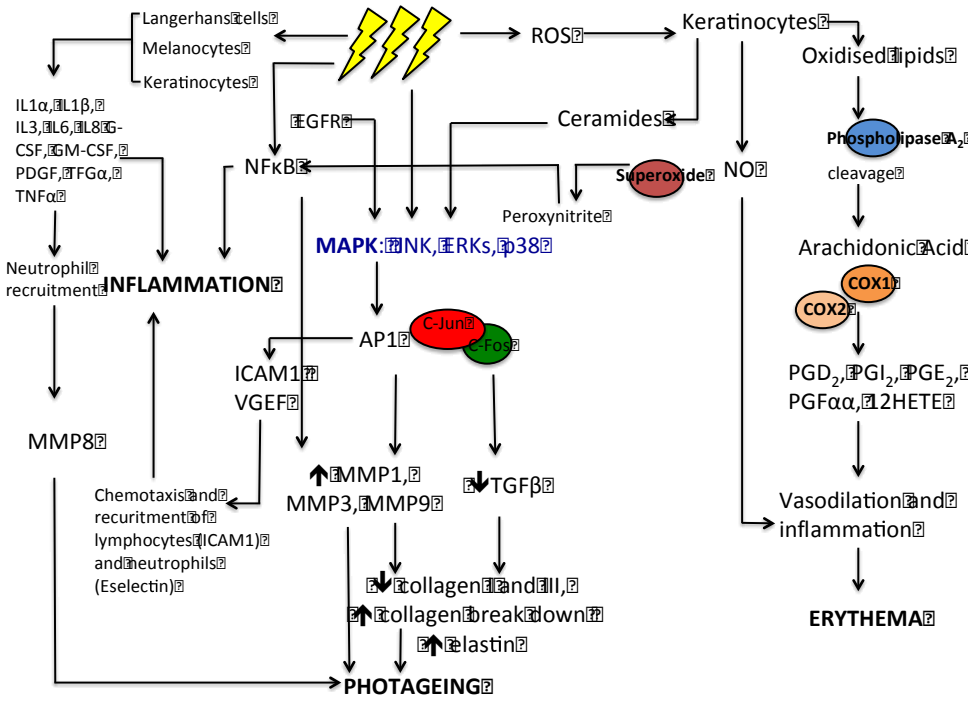


Figure 1.14 UVR signalling and gene regulation

1.7 Photoprotection

1.7.1 Sunscreens

Traditionally, sunscreen filters are categorized according to their optical properties; organic sunscreens absorb UVR, convert it to heat and prevent photons from interacting with skin molecules (Jansen *et al.*, 2013a) and inorganic (or physical) sunscreens which contain particles that reflect and absorb photons. Organic filters are aromatic compounds, and some of the earlier ones include Avobenzone and octinoxate (Jansen *et al.*, 2013a). They are invisible; hence aesthetically pleasing although UVR absorption may activate molecules leading to unwanted clinical reactions such as photoallergic contact dermatitis. According to an Australian study, adverse reactions from sunscreen ingredients can occur in up to 19% of sunscreen users (Foley *et al.*, 1993) and include allergic and irritant contact dermatitis,

phototoxic and photoallergic reactions, contact urticaria and in rare circumstances, anaphylaxis. More recent sunscreens however, such as Mexoryl SX (λ_{max} 345nm), Tinosorb (305nm, 360nm) and UVAsorb (312nm) are less likely to cause adverse effects.

Inorganic sunscreens are chemically inert and do not cause allergic sensitization, although they are often visible on the skin surface due to their scattering properties. They include zinc oxide and titanium dioxide. Generally, commercial formulations contain a mixture of both types of sunscreen (Tuchinda *et al.*, 2006; Yaar, 2007b) although none of these filters protect against visible and infrared radiation. There is an overlap in the way filters work as zinc and titanium oxides have absorbing as well as scattering effects, hence a more recent and probably more appropriate categorisation of sunscreens are into soluble and insoluble forms (Jansen *et al.*, 2013a; Osterwalder *et al.*, 2014).

The sun protection factor (SPF) is the ratio of the MED of protected skin over the MED of unprotected skin. The end point is cutaneous erythema assessed 24h after exposure to SSR, thus it is primarily used as a measure of UVB protection. A higher SPF affords greater sun protection and work done *in vitro* with an SPF 15, 30 and 60 demonstrates that after a given 10 minute exposure, 80% of a sunburn dose is reached using an SPF 15 sunscreen compared with 40% and 20% respectively for SPF 30 and SPF 60 (Osterwalder *et al.*, 2014; Sambandan and Ratner, 2011). In terms of transmittance, an SPF 60 sunscreen transmits half as much erythemal UVR as an SPF 30 sunscreen i.e. 1.7% compared to 3.33% (Osterwalder *et al.*, 2014). SPF is measured *in vivo* at 2mg/cm^2 although it is well known that the public use

sunscreens at much lower concentrations at 0.5mg/cm^2 (Lim and Honigsmann, 2007), therefore in such cases, the SPF provided by a labelled sunscreen is likely to be 4 times lower in practice. Analysis of the specified solar simulator spectrum used to determine SPF indicates that if 100% of the UVB is blocked, it is mathematically impossible to achieve an SPF in excess of approximately 11 without also blocking at least some UVA and that UVA protection needs to be provided in order for sunscreen products to achieve SPFs of 15 and higher (Sayre *et al.*, 2008) suggesting significant erythemal contribution from UVA. Practically complete blockage of UVB by means of the two most efficient UVB filters (5 % Octyltriazone (EHT) plus 10 % Iscotrizinol (DBT)) yields an SPF 14, whereas with the addition of only 4% bisoctrizole (MBBT) the SPF jumps to SPF 50.

Methods for testing UVA protection vary by country. In the EU, in order to give broad band cover; UVA-PF/SPF $\geq$ 1 /3 (Jansen *et al.*, 2013a) UVA cover should be at least 1/3 of the SPF. In the UK, the Boots 5 star rating, with 5 star providing a UVA/UVB ratio >0.9 provides closer to uniform UVB/UVA protection which may be regarded as ideal sunscreen performance (Hexsel *et al.*, 2008).

The US uses a different strategy to measure broadband sunscreen efficacy based on the critical wavelength (CW), defined as the wavelength at which 90% of the total area under the absorbance curve occurs. Sunscreens that have a CW of >370 nm claim broad-spectrum status (Jansen *et al.*, 2013a).

It has previously been unclear whether sunscreens are of any benefit in the

prevention of skin cancer. Some have argued that they may just serve to increase the amount of time users spend in the sun, subject them to larger doses of UVA and hence ultimately increasing skin cancer risk. This has been supported by a frequently cited study of university students who increased their time in the sun during a spring vacation while using a sunscreen with a high SPF (Autier *et al.*, 1999). However, a land mark randomized trial in Queensland Australia, showed that SPF15 broadband sunscreen users had 39% fewer SCC on head, neck, arms and hands compared to the control group (Green *et al.*, 1999) over a 4.5 year period. In a further 8-year follow-up there was nearly a 40% reduction in SCC (van der Pols *et al.*, 2006) and a 50% reduction in melanomas over a 15-year period (Green *et al.*, 2011).

1.7.2 Topical DNA repair enzymes

The use of DNA repair enzymes is an emerging approach to enhance DNA repair after UVR exposure. In the mid 1970s, the bacteriophage T4 DNA repair enzyme endonuclease V (T4N5) was found to augment NER in XP cells (Tanaka *et al.*, 1975). T4N5 catalyzes two reactions. It acts as a glycosylase, cleaving the glycosyl bond linking the base to the deoxyribose phosphate chain to leave an apyrimidinic/apurinic (AP) site. It has endonuclease activity which cleaves the phosphodiester bond leaving the 'free' phosphate backbone. Enzymes of the host cell then continue the process by removing the hanging CPD lesion by exonuclease activity and repair the site via polymerases (see Figure 1.15).

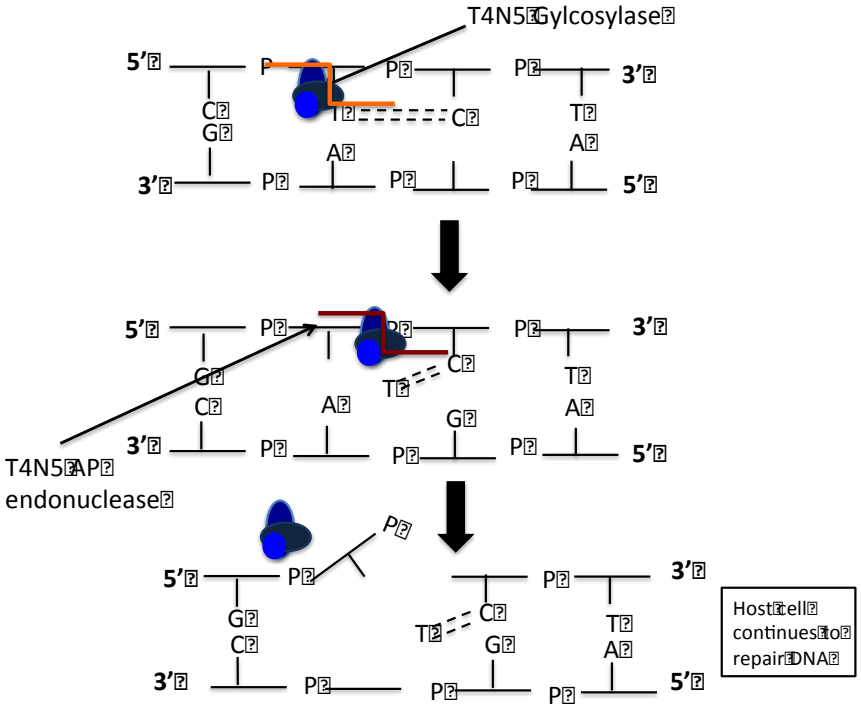


Figure 1.15 Repair initiated by T4N5 which has binding, glycosylase and endonuclease activity Adapted from (Friedberg and Wood, 1995)

The use of T4N5 to enhance DNA repair in the skin can be delivered via liposomes (lipid bilayer molecules) where T4N5 is encapsulated between membranes in order to facilitate delivery across the skin. T4N5 can be delivered to epidermal cell (in keratinocytes and Langerhans cells) cytoplasm within 1h (Yarosh, 2002) and is available as Dimericine®, Studies with these enzymes (Yarosh, 2002) show that CPD repair is associated with a reduction in MMP1 (Dong *et al.*, 2008), erythema, a decrease of immunosuppressive cytokines such as TNFα and IL10 (Wolf *et al.*, 2000) and thus the CPD triggers a series of biological processes It also reduces UVR immunosuppression. (Kripke *et al.*, 1992; Kripke *et al.*, 1996). Treatment with UVR prior to the application of a contact sensitizer such as dinitrochlorobenzene (DNCB) prevents the normal CHS response. However, topical application of T4N5 after UVR, but prior to sensitization results in a restored CHS response. T4N5 also

blocks UVR suppression of the DTH response and formation of T_{reg} cells (Kripke *et al.*, 1992; Wolf *et al.*, 1993) (see 1.9 The immune response to UVR). This has implications for skin cancer, numbers of which can be reduced in mice with the topical application of T4N5 (Yarosh *et al.*, 1992). In a landmark randomized study in XP patients, topical T4N5 was found to reduce the incidence of AKs and BCCs by >68% and 30% respectively (Yarosh *et al.*, 2001).

Photoreactivation is the term used to describe the repair of UVC and UVB induced DNA damage using long wave UVR, and in the presence of specific enzymes (Jagger, 1960) termed photolyases. These are found in non-placental mammals, as well fish, reptiles, prokaryotes and eukaryotes, although controversially the presence in human white blood cells (Sutherland and Bennett, 1995) has been suggested. DNA photolyases were first shown over 40 years ago to enhance DNA repair but it is only in the last few years have sufficient quantities been purified to permit detailed studies. Photolyases are specific to the type of DNA photolesion and there are CPD (class I and II) and 6-4PP photolyases (class III): (Weber, 2005). All photolyases have a flavin adenine dinucleotide (FADH) cofactor and a second chromophore. In class 1, the second chromophore is either a folate (MTHF) or deazaflavin (8-HDF) which absorbs at either 384nm or 438nm respectively (Sancar, 1990). The absorption maximum for flavin is at 313nm.

The non-covalently bound flavin cofactor plays the essential role in catalysis and in the fully reduced form: FADH-, acts as a 2-electron donor. The second chromophore absorbs near UV and visible wavelengths and transfers the excitation energy to FADH to yield the excited singlet state FADH-*. Electron donation then breaks up the cyclobutane ring of the CPD (Sancar *et al.*, 1987; Weber, 2005),

following which electron restabilisation returns it to the fully reduced FADH- cofactor. Other work (Sancar, 2003) suggests both chromophores absorb photoreactivating UVR/light and that as it has a higher extinction coefficient (more likely than flavin to absorb light at a given wavelength) the second chromophore acts to improve CPD repair by 10-1000 fold (Sancar, 2003).

Repair of CPD in the opossum (*Monodelphis domestica*) by photoreactivation reduced UVR induced erythema (Ley, 1985). This not only enabled an understanding of a biological role of CPD in the skin but also introduced the concept of manipulating biological processes through repair of DNA damage. Liposome encapsulated photolyase also has immunomodulatory functions and restores APC activity in mice (Vink and Roza, 2001), reduces UVR induced apoptosis and reduces the inhibition of CHS *in vitro* (Stege *et al.*, 2000). Using mice expressing a marsupial CPD photolyase transgene, the vast majority of acute responses in the UVR exposed skin (sunburn apoptosis, hyperplasia and mutations induction) can be ascribed to the CPD (Schul *et al.*, 2002) with a minimal contribution by 6-4PP (Jans *et al.*, 2005). CPD photolyase mice remained tumour free for longer than 6-4PP photolyase mice after multiple UVB doses. This work supports the CPD as the principal cause of NMSC. In humans, two main studies have looked at the impact of photolyase on CPD repair. Stege and coworkers (Stege *et al.*, 2000) showed that DNA photolyase repair reduced CPD by 40-45% after a 30-minute exposure to UVASUN (365nm, maximum peak). Photolyase also prevented UVR induced ICAM1 expression (important to recruit inflammatory cells to the skin) as well as prevented the suppression of the elicitation of hypersensitivity to nickel, sunburn cell formation and erythema.

Recently the addition of photolyase to topical sunscreens (SPF 50) has been shown to significantly reduce CPD 72h after 4 daily 3MED SSR exposures (Berardesca *et al.*, 2012). In terms of clinical endpoints recent work showed that over a 3 month period 6 patients had improvement in the number of AK with a topical application of a lotion containing photolyase (Puviani *et al.*, 2013), although this study did not have a control study population.

Plant derived, liposome encapsulated OGG1 enhances 8oxoG removal from human epidermal keratinocytes and recent work incorporating T4N5, photolyase and OGG1 as well as topical antioxidants into sunscreens had the effect of decreasing CPD in the skin (Emanuele *et al.*, 2014) but interestingly not 8oxodG. Of note certain sunscreens (phenylbenzimidazole-5-sulfonic acid (PBSA) appear to oxidise guanine *in vitro* following UVB irradiation (Bastien *et al.*, 2010).

1.7.3 Antioxidants

The skin contains many antioxidant enzymes (AOx): SOD, catalase and glutathione (GSH) peroxidase as well as non-enzymatic antioxidant molecules: α-tocopherol (vitamin E), L-ascorbic acid (vitamin C) and GSH. Topical AOx convert free radicals to less reactive molecules through pairing a single electron or by removing the unstable electron. For example SOD catalyses the conversion of O_2□- radicals into H_2O_2 and O_2, which is further reduced to H_2O_2 and O_2 with the aid of catalase and GSH peroxidase. However sub-erythemogenic UVR doses deplete tocopherol in human stratum corneum (Thiele *et al.*, 1998) resulting in increased oxidative stress leading to lipid peroxidation, protein oxidation (Sander *et al.*, 2002) and DNA

damage, which over time leads to photocarcinogenesis, photoageing and immunosuppression (Chen *et al.*, 2012). Work has examined the usefulness of exogenous antioxidants in mice. Oral AOx such as green tea (Elmets *et al.*, 2001) or grape seed extract (Jansen *et al.*, 2013b) is thought to play in role in reducing UVR-induced skin cancer formation. Topical green tea extracts (polyphenols) have also been shown to reduce DNA damage and p53 formation after PUVA therapy in reconstructed skin (Zhao *et al.*, 1999), probably mediated through IL12 dependent DNA repair and a subsequent reduction in skin inflammation (Meeran *et al.*, 2009; Schwarz, 2008).

Vitamin C (1% vitamin C, L-ascorbic acid)	Also found as ascorbyl phosphate and ascorbyl 6-palmitate yet these do not achieve same activity levels as ascorbic acid or vitamin C i*n vivo.* Vitamin C increases collagen synthesis and reduces MMP1 expression.
Vitamin E (α tocopherol acetate, α tocopherol succinate)	Multitude of animal and human studies show a reduction in lipid peroxidation, photoageing, immunosuppression and photocarcinogenesis
Vitamin A (retinoids (isotretinoin, tretinoin, tazarotene, carotenoids (βcarotene and lycopene)	Early animal studies suggested that retinyl palmitate (vitamin A- retinol derivative) had photocarcinogenic effects (Wang *et al.*, 2010b) although results were inconclusive and this derivative is used infrequently Carotenoids on the skin scavenge $^{1}02$ and quench lipid peroxidation
Tea polyphenols (epicatechin, epicatehin-3-gallate, epigallocatechin)	Tea polyphenols are unstable and activity diminishes quickly. Formations are stablished to reduce susceptibility to oxidation. Have anti-inflammatory and anticarcinogenic effects and can inhibit collagenase activity.
Plant antioxidant – caffeic acid (ferulic acid, phenethyl ester)	Found in topical preparations particularly in combination with L-ascorbic acid and α tocopherol.
Soy isoflavones	Contains genistein and daidzein. Diets high in soybeans are anticarcinogenic. Topical genistein decreases UVR induced oxidative damage, immunosuppression and inflammation.

Table 1.6 Common topical antioxidant preparations
Adapted from *Chen et al* (Chen *et al.*, 2012)

Thus there is a growing trend to incorporate non-enzymatic AOx into skin care products and sunscreens, specifically to combat photoageing. Two have been tested to penetrate the skin. Topical vitamin E has been shown to be particularly useful in reducing UVB induced sunburn cell formation in animal models whilst vitamin C (stable form; L-ascorbic acid) is particularly effective against PUVA induced phototoxic insults (Darr *et al.*, 1996). The protective benefit of combining these AOxs with sunscreens has been demonstrated in human studies. Participants received either a sunscreen with SPF 25 or the same sunscreen with a combination of caffeine, vitamin E, vitamin C, and after UVR, the sunscreen and antioxidant group had a 17% greater reduction in MMP1 levels compared with sunscreen alone (Matsui *et al.*, 2009). In another group using vitamin C and vitamin E, there was significant protection against MMP9 induction and pigment formation (Wu *et al.*, 2011).

Other effects include a reduction of erythema, sunburn cell formation (SBC) and a reduction in UVR immunosuppression likely due to a reduction in lipid peroxidation and migration of LC from the epidermis (Yuen and Halliday, 1997). Some animal studies also suggest vitamin E reduces the chronic effects of UVR on the skin particular skin cancer formation (Burke *et al.*, 2000). For a comprehensive review see Chen 2012 (Chen *et al.*, 2012).

1.8 The immune response

The skin is a mechanical defence barrier, but also has the capacity to induce an immune response via the skin associated lymphoid tissue (SALT) (Streilein, 1989). The immune response has innate and adaptive arms. The innate response is rapid

and its essential components include cytokines, complement, mast cells, microbial peptides and lymphoid derived natural killer cells as well as leukocytes: granular (neutrophils, eosinophils and basophils) and agranular (monocytes and macrophages). Leukocyte derived cytokines that exert effects on other white blood cells are called interleukins (IL), and those that have chemoattractant activity are termed chemokines and sub grouped as CXC or CC according to the position of the cysteine (C) or amino acid (X) residue. Induction of the adaptive immune system is critically dependent on cytokines, and is specific and enhanced with each successive antigen encounter due to T cell memory. It consists of humoral (B cell) and cellular (T cell) immunity.

Many cell types including B cells and macrophages are involved in antigen presentation or can reactivate memory lymphocytes. Dendritic cells (DC) are the only type of APC capable of initiating activation of naïve lymphocytes (CD45RA) upon their first encounter with antigen and are termed professional APC. These include epidermal LC (CD45+, CD1a+) and dermal dendritic cells (dDC). Exogenous antigen is internalized and processed by DC which migrate to draining lymph nodes. DCs (most literature indicate these as LC) to present antigenic peptide, in association with MHC class I or class II molecules, to T cells (Duthie *et al.*, 1999). According to the cytokine milieu and co-stimulatory molecules, T cells express either CD4+ or CD8+ with different functions. Memory T cells (CD45R0+) are also produced which remain in an inactive state until antigen is reencountered and a quicker and stronger immune response is mounted.

T_H1 cells

The major T_H1 derived cytokine is IFNγ that activates macrophages to kill intracellular pathogens (mycobacteria, fungi, protozoa) and stimulates natural killer (NK) cell cytotoxicity. Stimulated macrophages also release IL12 that continues to drive T_HO cells towards a T_H1 phenotype. The T_H1 arm facilitates cell mediated immunity and also contributes to the pathogenesis of autoimmune disease such as rheumatoid arthritis, multiple sclerosis, allergic contact dermatitis and psoriasis (see Figure 1.11).

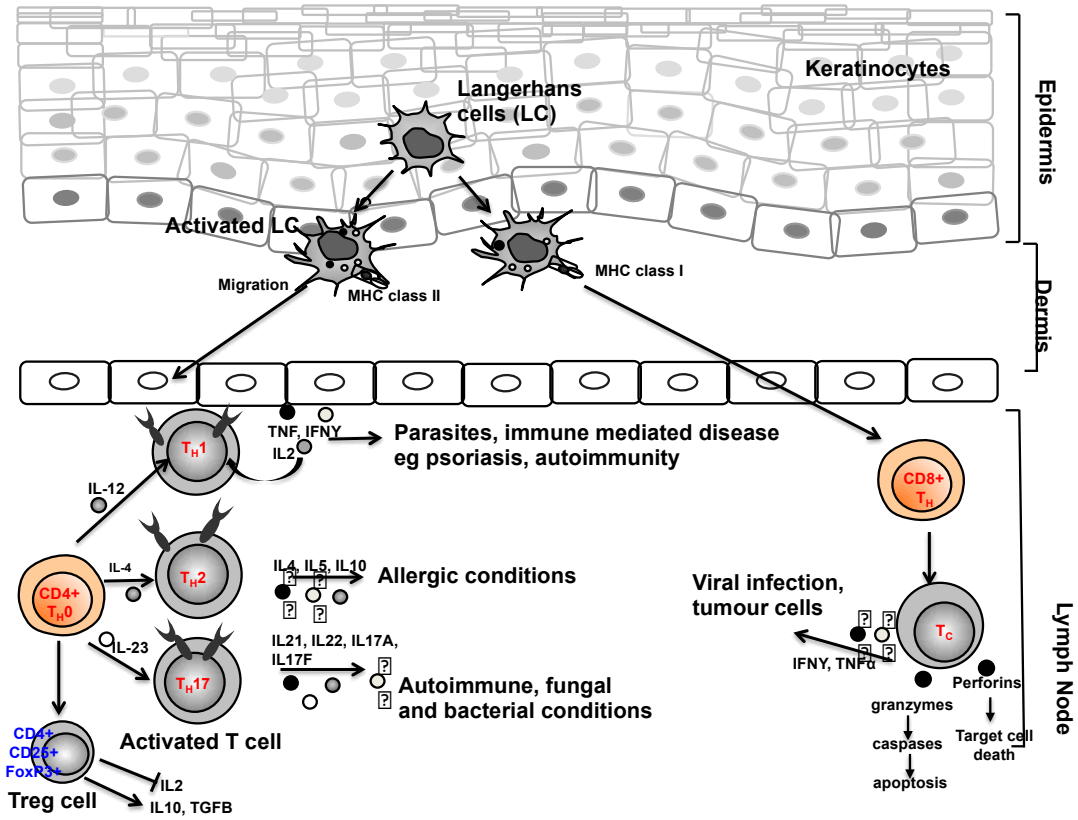


Figure 1.16 Cellular immunity
Stimulation of CD4+ T cells to differentiate to T_H1, T_H2 or T_H17 cells depending on cytokine milieu and via presentation of antigen with MHC class II and stimulation of CD8+ T cells via MHC class 1 to T_c cells.

T_H2 cells

T_H2 cells produce primarily IL4, IL5, IL6, and IL10, and are generally associated with allergic diseases. IL10 inhibits development of T_H1 responses.

T_H17 cells

CD4+ T cells that produce IL17, but not IFNγ or IL4, are referred to as T_H17 cells. They secrete IL17A, IL17F, IL21 and IL22, all of which are driven by IL23. T_H17 cells are rapidly induced in response to bacteria and fungi, and also have important roles in chronic inflammation and in mediating autoimmune reactions. They are upregulated in rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis where genetic studies have linked variants of the IL23 and IL23 receptor genes to susceptibility to psoriasis and psoriatic arthritis (Cargill *et al.*, 2007). In fact biological therapy with ustekinumab (a monoclonal antibody to p40 and a subunit of both IL12 and IL23) represents one of the most effective interventions for psoriasis (Leonardi *et al.*, 2008).

1.9 The immune response to UVR

Host immune defences have evolved to eliminate "non-self" which includes neoplastic cells. The first evidence demonstrating UVR immunosuppression emerged from the mid 1970s where highly antigenic skin tumours were rejected on transplantation to normal recipient mice of the same genetic background, however were not rejected and progressively grew when transplanted onto UVR irradiated recipients (Fisher and Kripke, 1977; Kripke and Fisher, 1976).

Depending on dose and wavelength, UVR markedly depletes epidermal LC, mostly inducing their migration to draining lymph nodes. High UVR doses may also trigger LC apoptosis (Schwarz *et al.*, 2000; Schwarz *et al.*, 2004), and some LC reaching the draining lymph nodes are CPD-positive (Sontag *et al.*, 1995). UVR suppresses the surface expression of adhesion and co-stimulatory molecules on migrating LC such as ICAM-1, CD80 and CD86, which impairs their antigen presentation capability to stimulate $CD4^+$ T_H1 cells, although the capacity to activate T_H2 cells remains intact (Simon *et al.*, 1990). Thus, there is a reduction of T_H1 cytokines (IL12 and IFNγ) and a switch to IL4 and IL10 cytokines. Various soluble immunomodulatory mediators are produced both locally and systemically (Clydesdale *et al.*, 2001; Duthie *et al.*, 1999) including pro-inflammatory (TNFα, IL1, IL6, IL8 and IL12) and anti-inflammatory (IL4 and IL10) cytokines. T and B cells, monocytes, macrophages and keratinocytes (de Vries, 1995) are activated to produce IL10, as well as PGE_2. Downstream production of PGE_2 as well as through UVR induced lipid peroxidation leads to a cytokine cascade also producing IL4 and IL10 (Shreedhar *et al.*, 1998). Histamine, platelet-activating factor (PAF) and neuropeptides (substance P, CGRP) are released which alter skin sensitivity explaining how the skin often feels slightly tender post UVR exposure (Harrison *et al.*, 2004). UVR stimulates the production of regulatory T cells (Treg) either natural nTreg produced in the thymus or iTreg, induced in the periphery which exert inhibitory functions through the release of cytokines (IL10, IL35, TGFβ), apoptosis of target effector T cells via release of granzymes and perforins, and sequestration of IL2 resulting in T_H1 anergy (Schwarz *et al.*, 1996).

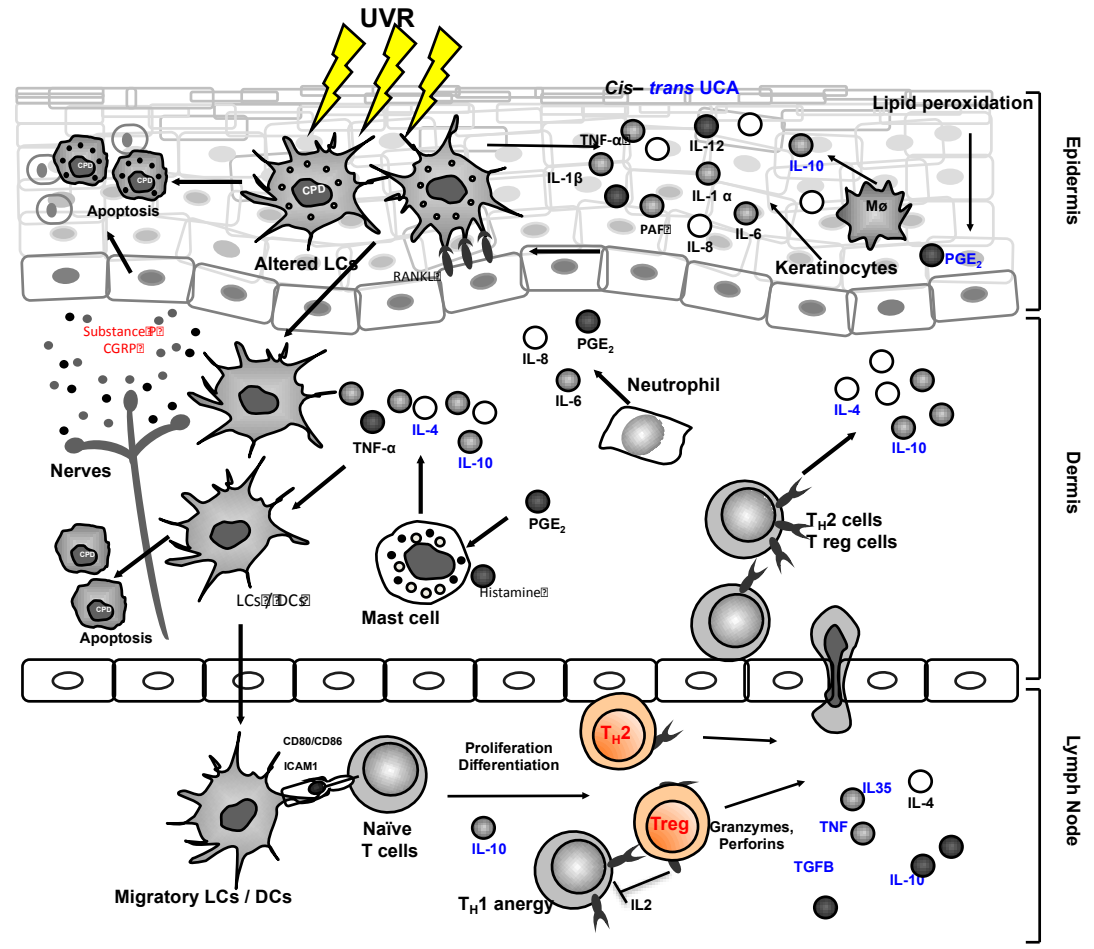


Figure 1.17 UVR induced immunosuppression
Migration of Langherhans cells (LCs) to the lymph nodes (LNs), reduction of T_H1 cells and a switch to IL10 and IL4 cytokines and a production of regulatory T cells (Treg) cells.

Measuring UVR immunosuppression

UVR induced suppression of acquired immunity in human skin is a normal phenomenon and can be clinically assessed using the CHS response to epicutaneous haptens (Kelly *et al.*, 1998; Moyal, 1998) or DTH responses to intradermally injected antigens (Moyal and Fourtanier, 2001, 2003). In the sensitisation phase, novel antigens are presented by LCs and other DCs to produce antigen specific memory T cells, and following subsequent re-exposure, the memory T cells

recognise those specific antigens and provoke a cutaneous inflammatory reaction, which is termed elicitation. Exposure of the skin to UVR prior to sensitisation induces impairment of antigen presentation and other immune changes that result in the suppression of elicitation. For instance, it has been shown that low doses of SSR, typically experienced after one hour of exposure to midday summer sunlight at mid-latitudes, can suppress the CHS response to 2,4- dinitrochlorobenzene (DNCB) in healthy humans (Kelly *et al.*, 2000). Exposures to doses of UVR that are only 30-50% of an MED suppress immunity in skin type I/II individuals (Kelly *et al.*, 2000). Therefore normal daily outdoor activities during spring and summer months will cause some degree of immunosuppression in a large proportion of individuals.

UVR can induce immune suppression both locally and systemically (Damian *et al.*, 1998; Kelly *et al.*, 2000; Moyal, 1998). Experimentally, local immunosuppression occurs when a sensitizing hapten/antigen is applied directly to the low UVR-irradiated site and is due to a depletion in the number of LC at the site of exposure (Toews *et al.*, 1980) although recently it has been suggested that LC exhibit regulatory activity (Beissert *et al.*, 2006; Schwarz *et al.*, 2000). Systemic (distal) immunosuppression, generally after a higher initial UVR dose is defined as the reduced immune responses detected when the sensitising hapten/antigen is applied to a distant non-irradiated site (Ullrich, 2005) . There is a reduced T_H1 response (Noonan *et al.*, 1981; Ullrich, 2005) and often a reduced immune response after application of the same hapten several weeks later (Toews *et al.*, 1980). This can be explained by stimulated keratinocytes releasing immunosuppressive soluble mediators, including IL10, TNFα (Ullrich *et al.*, 2007) and IL4 (el-Ghorr and Norval, 1997) (Hart *et al.*, 2000) in the microenvironment, preventing LC presenting antigen

to T_H1, as well as an induction of tolerance mediated via hapten specific Treg cells (Beissert *et al.*, 2006).

Immunosuppression can also be mediated by UCA (see section 1.4.1.2) as well as DNA damage. In fact, repair of UVR induced DNA damage also inhibits UVR induced immunosuppression in humans (Kripke *et al.*, 1992; Nishigori *et al.*, 1996) and induction of Tregs. Inhibition of UVR induced NO also appears to prevent UVR immunosuppression in human skin (Kuchel *et al.*, 2003).

It is well known that both UVA and UVB contribute to sunlight induced immunosuppression (Damian *et al.*, 1999; Poon *et al.*, 2005) with suggestion that sunlight is more immunosuppressive than either waveband alone (Poon *et al.*, 2005) and that UVA may protect the immune system from the suppressive effects of UVB (Reeve *et al.*, 1998; Reeve and Tyrrell, 1999). With UVB, increased levels of immunosuppression occurs with increasing dose however UVA has a bell shaped dose response curve so that after the peak immunosuppressive dose has been reached higher doses actually cause lower levels of immunosuppression until a dose is reached that is no longer suppressive (Byrne *et al.*, 2006). This complicates our understanding of UVA immunosuppression. Recent characterisation of the immunosuppression action spectrum using induction of nickel CHS model produced a peak at 370nm corresponding to UVA1 (Damian *et al.*, 2011) although the overal major immunosuppressive peak is in the UVB waveband.

Several studies have reported that organ transplant patients undergoing immunosuppressive therapy have an elevated risk of both NMSC and MM especially if they have a history of high sun exposure (Euvrard *et al.*, 1997). Drugs such as

azathioprine also cause DNA photodamage on UVR exposure, which probably plays a significant role in skin cancer induction (Kelly *et al.*, 1989; O'Donovan *et al.*, 2005; Yoshikawa *et al.*, 1990).

Pathological failure in UVR induced immunosuppression causes an abnormal inflammatory response to sunshine as seen in clinical conditions such as polymorphic light eruption (PLE) where following UVR exposure (primarily UVA (Ortel *et al.*, 1986)) there is in a DTH type response to a UVR unknown neoantigen (see 1.10.2 Photodermatoses), and a lack of LC migration and T_H2 mediated immunosuppressive cytokines (IL4, IL10) (Kolgen *et al.*, 2004; Kolgen *et al.*, 1999).

1.10 UVR and Dermatology

1.10.1 Phototherapy

Over the centuries, dating back from as early as 1400BC, sunlight has been used to treat skin diseases. Heliotherapy, named after the Greek god of the sun *Helios*, was used in ancient Egypt, Greece and Rome, but it wasn't until the second half of the 19th century that the use of UVR moved from being anecdotal to a more focussed form of therapy (Roelandts, 2007). During World War I, heliotherapy provided benefit to soldiers with traumatic leg ulcers and was also used in thermal stations to treat tuberculosis. There are reports of Florence Nightingale protesting against the orientation of the Royal Victoria Hospital in Southampton, UK, after observing that no sunlight could enter its wards. By the end of the 19th century, Niels Finsen used sun exposure in a more standardized way for treatment of Lupus Vulgaris. He was the first to make a switch from heliotherapy with natural sunlight to phototherapy

with artificial lamps providing a specific UVR output and won the Nobel Prize in 1903 for his work. Photochemotherapy has had a long history with the use of psoralen containing plant extracts (*Psoralea corylifolia* and *Ammi majus*) and sun exposure, to treat vitiligo dating from as far as 2000BC (Pathak and Fitzpatrick, 1992). These extracts have now been isolated and used as oral/topical/bath 8 methoxypsoralen (8MOP), oral 5-methoxypsoralen (5MOP) or bath trioxsalen with UVA (PUVA: peak emission 352nm) irradiation for treatment of vitiligo, psoriasis and other proliferative diseases. Notable improvement has been seen in psoriasis and the action spectrum for the phototoxic reaction lies in the 330-360 nm range (Plewig *et al.*, 1978) although earlier studies show peak photosensitization at 320-340nm (Cripps *et al.*, 1982). PUVA is marred with a series of side effects related to the agent including nausea as well as increased photosensitivity to sunlight during therapy causing burning, itching and an increased risk of skin cancer (Stern, 2001; Stern *et al.*, 1998). Considerable research in the 1980s went into developing alternative sources of phototherapy. Broad band UVB although considerably less efficient than PUVA for psoriasis was useful, but it wasn't until the identification of a peak at 313nm in the action spectrum for psoriasis clearance (Parrish and Jaenicke, 1981) that changed phototherapy practice. Narrowband (NB) UVB TL01 phototherapy (311–313 nm) has largely replaced broadband UVB although PUVA is probably more effective for treatment of psoriasis in the UK (Ling *et al.*, 2016). Other types of phototherapy that have been developed include extracorporeal photophoresis (Zic *et al.*, 1992) for cutaneous T cell lymphoma (CTCL) and chronic graft-versus-host disease (GVHD), topical photodynamic therapy with visible light for actinic keratoses and superficial BCC (Ericson *et al.*, 2008) and UVA1

phototherapy. The rationale for the development of the latter was the need for a unique high output mercury lamp (Mutzhas *et al.*, 1981; Plewig *et al.*, 1978) that emits biologically effective UVA1 doses and reduces the adverse effects of sunburn with the UVA2 part of the spectrum. Initial work for its use in acne and vitiligo proved unsuccessful and it was primarily used as a diagnostic tool for photoprovocation of UVA sensitive photodermatoses such as PLE. It was initially considered to have no adverse effect on tissue due to normal histology findings 72h after a single 60J/cm^2 dose (Mutzhas *et al.*, 1981), however it wasn't until the early 1990s, its therapeutic potential in disease was recognised. Krutmann and colleagues reported that exposure to high doses of UVA1 was beneficial for patients with severe acute atopic dermatitis (AD) (Krutmann *et al.*, 1992; Krutmann *et al.*, 1998). Strongest evidence now exists for its use in localised scleroderma (Andres *et al.*, 2010), systemic lupus erythematosus (SLE) as well as acute AD. However, treatment depends on the most convenient strategy to implement and in many centres the UK, UVA1 is first-line for localized scleroderma only. There are no internationally agreed definitions but 10–29 J/cm^2 is generally considered 'low dose', 30–59 J/cm^2 'medium dose' and >70J/cm^2 'high dose' (Kerr *et al.*, 2012a). Established practice at St John's Institute of Dermatology is medium/low dose therapy, and as a general rule, one treatment course does not exceed 30 exposures once or twice a year (Krutmann and Akimichi, 2007). It is advised that immunosuppressive drugs such as azathioprine are not combined with UVA1 (Brem and Karran, 2012) and in children its use is limited for only severe cases of scleroderma where no other treatment options exists. The high output metal halide lamps (Sellamed 4000W, Sellas Medizinische Geräte GmbH, Ennepetal, Germany)

deliver biologically effective UVA1 at high doses (Gambichler *et al.,* 2013), yet despite this, exposure times are long (often 45 minutes) (von Kobyletzki *et al.*, 1999) which patients may find uncomfortable, in particular due to the heat generated from infrared emission. The use of cold light lamps offering UVA1 without infrared have also been assessed (Polderman *et al.,* 2005; von Kobyletzki *et al.,* 1999) but outputs are lower and thus treatment times longer. Other adverse events include hyperpigmentation: particularly unwanted in darker skinned individuals and limits its use in this subpopulation, erythema and pruritus; likely due to mild PLE photoprovocation (Beattie *et al.,* 2005a). Activation of herpes infections have also been noted following UVA1 therapy (Krutmann and Morita, 1999). To reduce the risk of precipitating a UVA sensitive photodermatoses or photosensitive AD, UVA1 photosensitivity testing is performed on all patients prior to starting therapy although this is not standard practice in Germany (Gambichler *et al.*, 2013).

The use of UVA1 phototherapy can be split into 4 main categories based on pathogenesis (Krutmann and Akimichi, 2007). All effects are likely mediated by its generation of $^{1}O_2$ (Morita *et al.*, 1997) causing T cell apoptosis (Krutmann and Morita, 1999), MMP1 upregulation (which is also mediated through inflammatory markers IL6/IL1β) and decreased mast cell and LC numbers and function (Breuckmann *et al.*, 2002; Grabbe *et al.*, 1996) see Table 1.7.

Disease	Method of Action	Supporting Evidence
T cell mediated Disease Acute Atopic Dermatitis (AD) CTCL	ROS mediated apoptosis of skin infiltrating T cells (Morita *et al.*, 1997), decreased IFNy, reduction in LC (Ig E binding) and mast cells in the dermis (Grabbe *et al.*, 1996), and a decrease in CD4+ CD1a dendritic cells and activated EG2+ eosinophils (Breuckmann *et al.*, 2002).	For acute AD (Krutmann *et al.*, 1992; Krutmann and Schopf, 1992)—UVA1 medium dose more effective than NBUVB and PUVA and high dose used in Germany (Krutmann *et al.*, 1998) although for most centres, expense of high output UVA1 apparatus is not justifiable. Inferior to NBUVB and PUVA (Tzaneva *et al.*, 2010) for chronic AD although no studies comparing acute and chronic AD (Kerr *et al.*, 2012a)
Connective Tissue Disease Localised scerloderma Systemic sclerosis (SS)	Increased synthesis of MMP1 (Jinnin, 2010) to break down fibrotic lesions (caused by increased type 1 and III collagen) causing softening of plaques and a lack of new collagen synthesis through TGFB down regulation (Breuckmann *et al.*, 2004; Gambichler *et al.*, 2013).	Medium (Sator *et al.*, 2009; Su *et al.*, 2011) and high doses (Stege *et al.*, 1997; Su *et al.*, 1998) effective for localised scleroderma and medium doses has been shown to be more effective than NBUVB (Kreuter *et al.*, 2006) and improves function especial joint movement. Medium dose softens fibrosis in systemic sclerosis (SSc) (Morita *et al.*, 2000), improves microstomia (Tewari *et al.*, 2011a), Raynaud's syndrome (Comte *et al.*, 2009) and low dose is useful in acrosclerosis (Kreuter *et al.*, 2004; Rose *et al.*, 2009). Improvement also noted with PUVA (Morita *et al.*, 1995; Scharffetter-Kochanek *et al.*, 1995).
Mast cell mediated skin disease Urticaria Pigmentosa	Reduced density, number and function of dermal mast cells (Grabbe *et al.*, 1996),	No relapse after 2y following high dose UVA1. Relapse with PUVA after 5-8 months (Gobello *et al.*, 2003; Stege *et al.*, 1996)
Phototherapy in HIV positive patients Psoriasis/HIV+ve	Likely decrease in T cell mediated disease.	Compared to UVB, UVA1 does not affect HIV viral load. Whole body UVA1 effective(Breuer-McHam *et al.*, 2001)
Miscellaneous Keloid and hypertrophic scars Pityriasis rubra pilaris (PRP) Lupus erythematosus	MMP1 activity on collagen over-production	Marked softening, flattening and reappearance of normal looking collagen and elastic fibres (particularly good for large scars and burn scars) (Asawanonda *et al.*, 1999) Use in PRP (Herbst *et al.*, 2000) Low dose UVA1 for lupus, decrease in clinical indices as well as titres of anti-SSA/ANA abs (McGrath, 1994; McGrath *et al.*, 1996). Risk of PLE provocation

Table 1.7 Use of UVA1 phototherapy in skin disease
NB this table is not comprehensive

Caution needs to be exercised due to the potential increased risk of skin cancer and photoageing however there is a lack of studies examining the long-term effects of multiple UVA phototherapy courses on the skin.

1.10.2 Photodermatoses

The photodermatoses are skin disorders in which an abnormal pathological response is precipitated by normal exposure to sunlight and/or artificial sources of UV and/or visible radiation. An adapted classification system based on our experience at our tertiary photodermatology clinic at St John's Institute of Dermatology, London of patients presenting to clinic over a 6-year period (2006-2012) is shown in Table 1.5.

Category	Photodermatoses	Number	% of total
Immunologically Mediated	Polymorphic Light eruption (PLE)	337	27
	Chronic Actinic Dermatitis (CAD)	204	16
	Solar Urticaria (SU)	66	5
	Actinic Prurigo (AP)	48	4
	Hydroa Vacciniforme (HV)	6	0.5
Drug and Chemical Induced			
Exogenous	Phototoxicity	16	1
	Photoallergic contact dermatitis	18	1
Endogenous: (cutaneous porphyrias)	Erythropoietic protoporphyria (EPP)	68	5
	Porphyria cutanea tarda (PCT)	29	2
	Congenital erythrpoeitic porphyria (CEP)	6	0.5
	Variegate porphyria (VP)	4	<0.5
Undiagnosed		24	2
Other		72	6
Defective DNA repair	Xeroderma Pigmentosum (XP)	76	
	Total	**1274**	100

Table 1.8 Classification of photodermatoses

Photosensitivity patterns of some of the most common photodermatoses (PLE, SU, EPP, drug induced, XP) at St John's Institute of Dermatology photodermatology

department are shown in Table 1.8. CAD tends to persist through out the spring/ summer period with intermittent flares and is not included in the figure below.

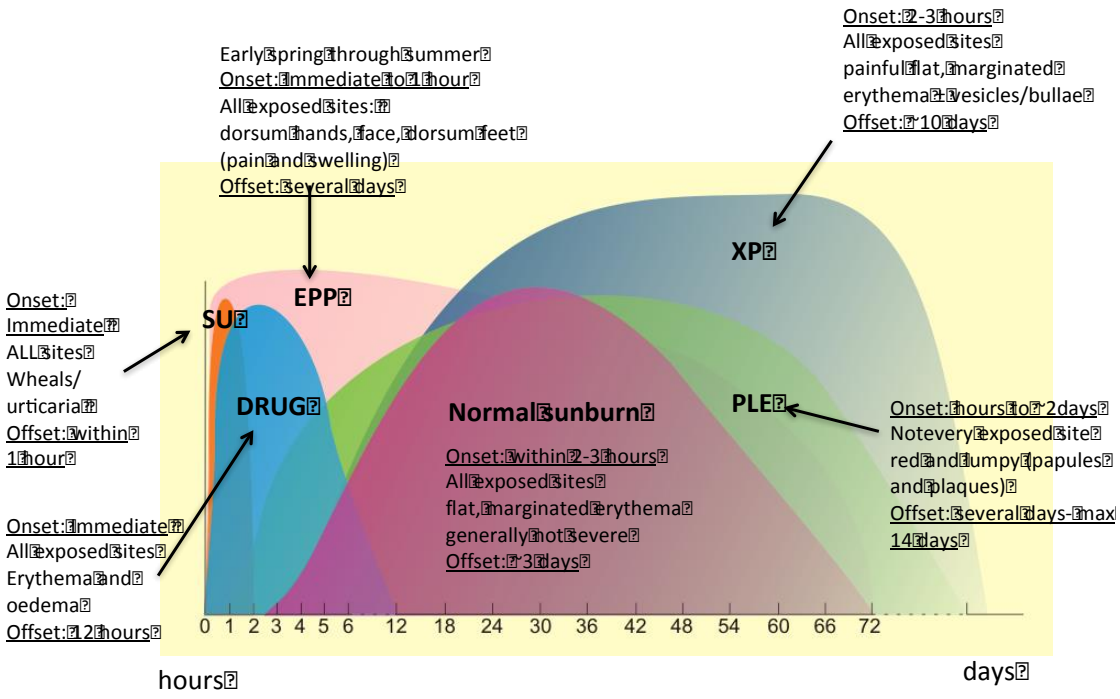


Figure 1.18 Photosensitivity patterns in common conditions presenting to a photodermatology clinic
Adapted from data kindly given by Dr D McGibbon.

PLE is the most common photodermatosis, and is characterised by an itchy erythematous papulo-vesicular rash, which often develops the day after sun exposure, and resolves within a few days or weeks. Its prevalence is 15% in the UK (London 51.5°N) (Pao *et al.*, 1994) and 5% in Perth (32.0°S) which is explained by its primarily UVA provocation (Janssens *et al.*, 2007) and is seen more commonly at temperate latitudes where the ratio of UVA:UVB is primarily weighted towards UVA.

1.11 Aims of the thesis

Most work in photobiology has focussed on non-terrestrial UVC, and UVB. Recent data suggest that UVA may have more potential biological consequences in human skin than previously thought, although these concepts are based on *in vitro* work in mouse and explant human skin. Understanding the carcinogenic and other adverse effects of UVA in human skin *in vivo* has important public health concerns, particularly as UVA1 is used for phototherapy in the UK and abroad for treatment of predominantly dermal skin diseases. Furthermore, UVA1 is the main spectral source in tanning lamps and represents over 75% of the environmental exposure from terrestrial UVR.

The main goals of this thesis were to assess the contribution of UVA1 to the molecular and cellular effects of sunlight in the skin *in vivo*. Studies with UVA1 were done along side those with UVB, because the latter has known effects and can act as a positive control. Action spectra (see section 1.3.2) typically show that UVB is several orders of magnitude more effective than UVA, so skin exposures were based on erythemally equivalent doses rather than physical measures of exposure (e.g. J/m^2).

The specific aims of the PhD studies were to:

- Evaluate the production and repair of DNA photodamage in the epidermis and dermis. The endpoints for this study were the thymine (TT) dimer, which is a type of cyclobutane pyrimidine dimer (CPD) and the 6-4 photoproduct (see Chapter 3).

- Compare CPD repair in melanocytes and basal epidermal keratinocytes (see Chapter 4).
- Assess UVR-induced changes in gene expression by micro array and validate selected gene changes using RNA and protein expression/function studies (see Chapter 5).
- Assess downstream DNA damage and other cellular changes in the skin following application of topical repair enzymes (see Chapter 6).

In each chapter the impact of research on human health is discussed. These studies resulted in 4 publications, which are included at the end of the thesis. One of the publications was subject to a commentary in the Journal of Investigative Dermatology and this is also included. 3 other publications, unrelated to the thesis, are included to demonstrate the scope of my interest in photodermatology.

Chapter 2 Materials and methods

2.1 Volunteer recruitment

The study was approved by the St Thomas' Hospital, London, UK, Ethics Committee (REC no: 09/H0802/98) and conducted in accordance with the Declaration of Helsinki Principles. The details of all healthy skin type I/II (Fitzpatrick, 1988) volunteers studied in are shown in Table 2.1. All volunteers gave written informed consent.

Volunteers	Age	Sex	Skin[a] type	UVA1 MED J/cm^2	UVB MED mJ/cm^2	Analysis Performed
DNA damage dose response study (Chapter 3)						
001	28	F	I	48.8	30.0	IF[b]
002	25	F	I	48.8	30.0	IF
003	20	M	II	48.8	30.0	IF
004	21	F	II	53.0	30.0	IF
005	22	F	I	48.8	23.0	IF
006	34	M	I	61.1	19.0	IF
DNA repair time course study (Chapter 3)						
001	28	F	I	31.3	19	IF
002	28	F	I	31.3	30	IF
003	22	M	I	48.8	23	IF
004	24	F	II	31.3	30	IF
005	23	M	I	48.8	23	IF
006	23	F	II	48.8	23	IF
Apoptosis and DNA repair study (Chapter 4) *Volunteers taken from DNA repair time course study (Chapter 3) and protein validation study (Chapter 5*)						
001	28	F	I	31.3	19	IF
002	28	F	I	31.3	30	IF
003	22	M	I	48.8	23	IF
004	24	F	II	31.3	30	IF
005	23	M	I	48.8	23	IF
006	23	F	II	48.8	23	IF
001	24	F	II	61.1	37	IF
002	27	F	II	61.1	37	IF
003	22	F	I	48.8	30	IF
Gene time course study (Chapter 5)						
001	21	M	II	48.8	30.0	Microarray, RTqPCR[c]
002	27	M	I	48.8	30.0	Microarray, RTqPCR
003	21	M	I	48.8	3 0.0	Microarray,

						RTqPCR
004	21	F	I	61.8	37.0	Microarray, RTqPCR
005	21	F	I	61.8	23.0	Microarray, RTqPCR
Gene dose response and UVA1 and UVB comparison study (Chapter 5)						
001	20	M	I	61.1	19	Microarray, RTqPCR
002	21	M	I	61.1	23	Microarray, RTqPCR
003	23	F	II	61.1	30	Microarray, RTqPCR
004	22	F	I	61.1	23	Microarray, RTqPCR
005	28	F	II	76.2	30	Microarray, RTqPCR
Protein validation study (Chapter 5)						
001	24	F	II	61.1	37	IF
002	27	F	II	61.1	37	IF
003	22	F	I	48.8	30	IF

Table 2.1 Demographics for all volunteers used in the study
[a] Fitzpatrick skin type (see Introduction Chapter 1)
[b]Immunofluorescence
[c]Reverse transcription quantitative PCR

2.2 UVR sources and dosimetry

The UVA1 irradiation source was a Sellamed 24000 System Dr Sellmeier (Sellas Medizinische Geräte GmbH, Gevelsberg, Germany). Irradiance was measured prior to each volunteer irradiation with a radiometer (Model IL1400A, International Light Technologies, Peabody, MA) and was typically ~74mW/cm^2 at a 24.5cm distance. Narrowband UVB (300nm) was produced by a monochromator (Oriel, Irvine, CA). This was a 1-kW xenon arc; grating blazed at 250 nm: slits set for 3nm full width at half maximum bandwidth. UVB was delivered with a liquid light guide (Oriel), with an exit diameter of 5mm in direct contact with the skin. Irradiance (~0.5 mW/cm^2) was measured with an SEL623 thermopile (International Light Technologies) attached to an IL1400A radiometer (detector and meter calibrated by the United Kingdom Accreditation Service-accredited Guy's and St Thomas' Hospitals Trust

UVR laboratory).

SSR was generated by a 1-kW xenon arc solar simulator (Oriel Corp., Didcot, Oxfordshire) giving an even field of irradiance (290–400 nm) of ~15 mW/cm^2 on the skin surface when 11 cm from the source. Irradiance was routinely determined with a wide band thermopile radiometer (Medical Physics, St Thomas' Hospital, London).

Emission spectra and irradiances of the UVA1, UVB and SSR sources were checked with a DM150BC double-monochromator spectroradiometer (Bentham Instruments, Reading, UK) using an integration sphere and gratings blazed at 250 nm.

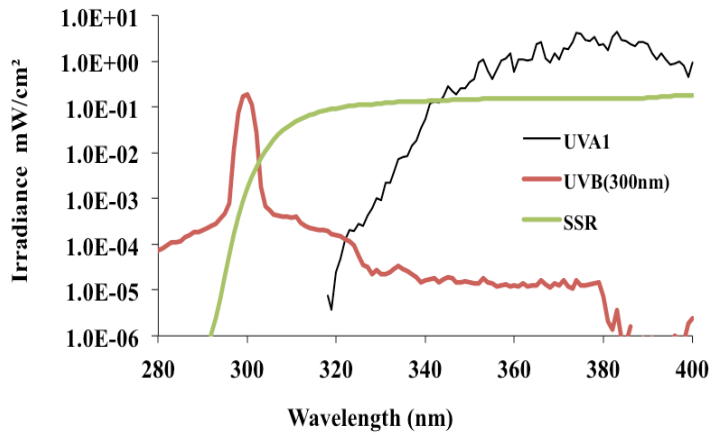


Figure 2.1 Emission spectra for UVA (300nm), UVA1 and SSR
The UVA2 (320–340 nm) content of the UVA1 source was 0.2%, which is equivalent to 0.6% of the overall erythemally effective energy (EEE) when the emission spectrum is weighted with the action spectrum for erythema (CIE, 1998) (calculations not shown). For the SSR source, 6.8% UVB corresponds to EEE of 88%.

2.3 Irradiation protocol and assessment of erythema

To determine the MED, eight areas each measuring 1cm^2 on the left buttock were exposed to doses ranging from 15 to 74J/cm^2 for UVA1 and 12 to 58mJ/cm^2 for

UVB in 25% increments. These starting doses were based on advice from our clinical unit. A template made of leatherette (John Lewis, UK) with 8 x 1cm^2 areas was used to define the area of irradiation. The dose that caused just perceptible (jp) erythema at 24h (jpMED) was used, as it has been shown to provide a more reliable threshold end point than 'erythema with a definite border' (Ouinn *et al.*, 1994). An erythemal response to UVA is biphasic with an early phase due to heat emitted the UVA1 source, which fades rapidly (Diffey *et al.*, 1987) and peak UVA1 erythema occurring 8h post irradiation but the time course of erythema is broad and the peak often extends to 24h post irradiation (Beattie *et al.*, 2005a) with the reaction either fading or being replaced by pigmentation at subsequent time points (Ibbotson and Farr, 1999. With UVB, time to maximal erythema occurs 8-12h post irradiation (Farr *et al.*, 1988) with other studies showing that maximal erythema is reached 24h post UVB (Man *et al.*, 2003). Readings are generally carried out at 24h for practical reasons and in this thesis exposed sites were visually assessed by one clinician (AT) according to the following criteria: 0, no erythema; ±, observer not sure; 0.5, just perceptible erythema; 1, erythema with a definite border; 2, intense erythema ± papular response at 24 hours. Reflective spectroscopy was carried out 3 times per test site per volunteer, and the mean value was used to quantify erythema (Dia-Stron, Andover, UK). The difference between the mean measurement from each test site and an adjacent non-irradiated control site is the Δ erythema index value (ΔEI) and generally at 1MED this difference is approximately 60.

2.4 Volunteer irradiations

Our studies were based on fixed doses determined from the MED in the first 3 individuals: mean values for 1MED was 30.0±0 (SD) mJ/cm^2 for UVB (300nm) and 48.8±0 (SD) J/cm^2 for UVA1 which we defined as the 'notional MED' see Table 2.1. Volunteers were given fixed increments of this as shown in Table 2.2. For the gene studies, the UVA1 dose given was 50J/cm^2 (1MED), UVB at 30 mJ/cm^2. The actual MED of all individuals was within 2 increment of the notional MED. This approach was used rather than an individualised dosing regime because it was thought that fixed doses would reduce inter-personal variation.

Studies:	**DNA damage dose response study**			**DNA repair time course study**	**Gene time course study**	**Gene dose response study**			**UVA1 and UVB comparison study**	**Protein validation Study**
Approx. MED	**~0.5**	**1.5**	**3**	**3**	**~1**	**~¼**	**~½**	**~1**	**~1**	**~1**
UVA1 (J/cm^2)	24.4	73.2	146.4	146.4	50	12.5	25	50	50	50
UVB (mJ/cm^2)	15	45	90	90	-	7.5	15	30	30	30
Time point[a]	Immediate			Immediate, 3, 6, 24, 48h	6h and 24h	6h			6h and 24h	10h and 24h

Table 2.2 Doses of UVA1 and UVB given in all studies

[a]Unirradiated control biopsy was also taken in each study from each volunteer

The biopsy protocols are shown diagrammatically in Figure 2.2 (1MED UVB =30mJ/cm^2 and 1MED UVA1 =~50J/cm^2).

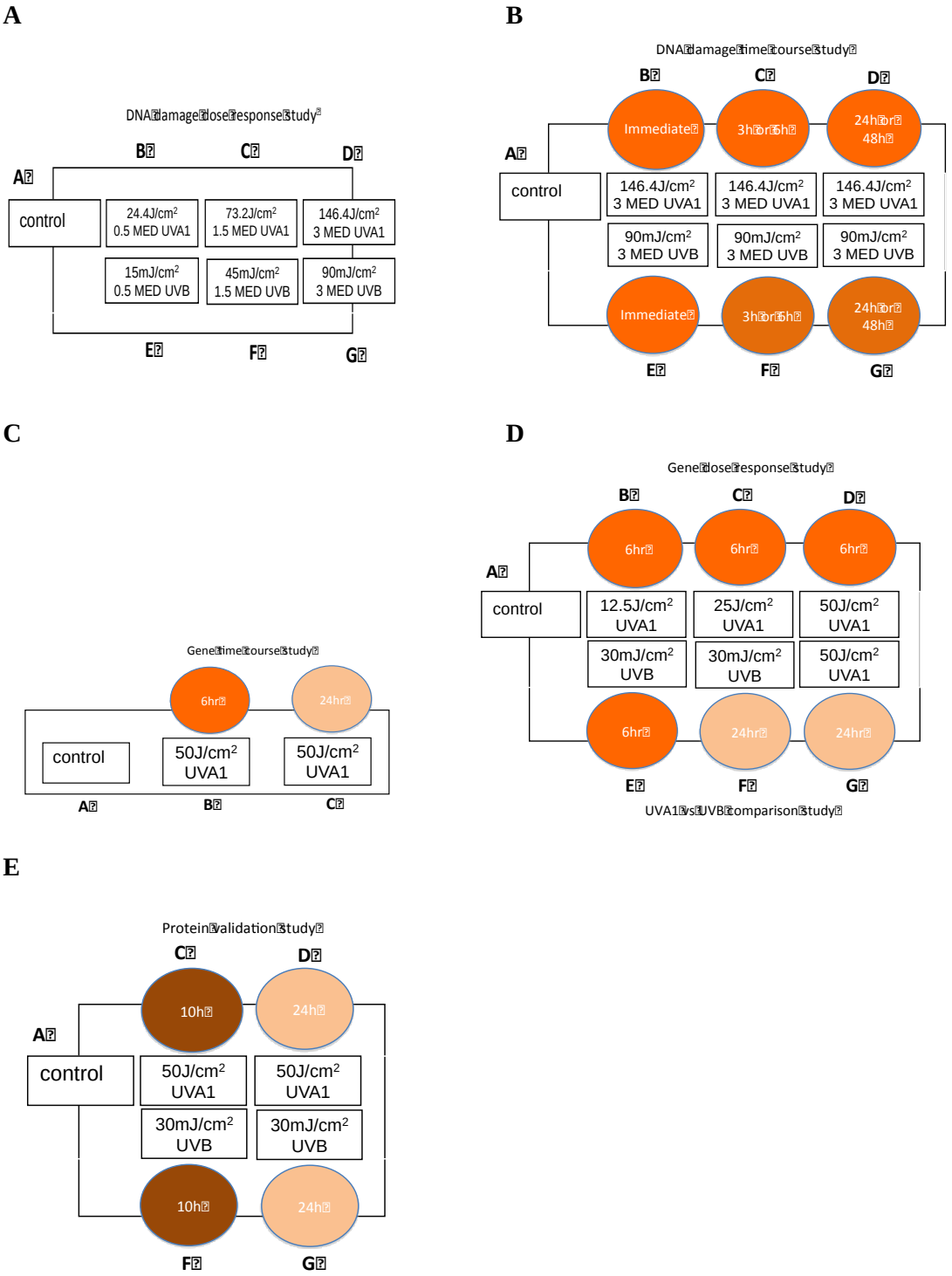


Figure 2.2 Irradiation and biopsy protocols for all studies (A-E)
NB: Lettering system enabled identification and tissue section labelling.

For the DNA repair time course study, due to the limit on the number of biopsies that could be taken on the buttock (maximum 7 on one site) there was a spread of time points. These are shown in Table 2.3.

Volunteer	Time post irradiation that biopsies were taken
UVA52-A2-001	Control[a], UVA1:0h, 24h, 48h UVB :0h, 24h, 48h
UVA52-A2-002	
UVA52-A2-003	Control[a], UVA1:0h, 3h, 24h UVB :0h, 3h, 24h
UVA52-A2-004	
UVA52-A2-005	Control[a], UVA1:0h, 6h, 24h UVB :0h, 6h, 48h
UVA52-A2-006	

Table 2.3 DNA repair time course study: biopsy time points
[a] control unirradiated biopsy taken at the immediate time point

2.5 Skin biopsy processing

2.5.1 DNA studies (paraffin embedded)

4mm punch biopsies were performed in a clinical setting (St Thomas' Hospital Outpatients Surgery room) under local anaesthetic (1% xylocaine with 1:200000 adrenaline, AstraZenaca, UK). Biopsies were fixed in 10% neutral buffered formalin (4% formaldehyde) for ~16 hours, followed by alcohol dehydration steps (50%, 70%, 90%, 100% ethanol in distilled water) and were then embedded in paraffin. Sections were cut at 4μm and placed on VWR superfrost® slides to ensure firm electrostatic attraction of formaldehyde-fixed paraffin sections.

2.5.2 Gene studies (RNA extraction)

All biopsies were immediately placed in cryovials and snap frozen in liquid nitrogen (~80°C). RNA was extracted using Trizol (Invitrogen, UK), quality checked via the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Waldbronn, Germany) and a RNA integrity number (RIN) was generated. This value is calculated using a

specific algorithm, which takes into account the 28S/18S peaks and any unexpected peaks in the 5S region that affect purity. A RIN of 10 indicates very high quality and according to published data (Fleige and Pfaffl, 2006) RIN >6 is of sufficient quality for gene expression profiling experiments. All RIN numbers in the gene study were between 7.6 and 9.6.

2.5.3 Protein validation (frozen)

Biopsies were embedded in OCT (VWR chemicals, Leuven, Belgium), cooled in Isopentane (VWR chemicals, Fontenay-sous-Bois, France), placed in cryovials and then frozen in liquid nitrogen. 5-7μm sections were mounted on Superfrost® plus slides and stored at -80ºC.

2.6 Immunostaining

Immunofluorescence (IF) was performed on paraffin and frozen sections. Immunohistochemistry (IHC) was performed on paraffin embedded samples.

2.6.1 Slide preparation

First, paraffin sections were placed in xylene (VWR Chemicals, Fontenay-sous-Bois, France) for 5 minutes followed by graded concentrations of ethanol for 2 minutes each (100%, 90%, 70% 50%) and washed in MilliQ distilled water (Millipore, MA, USA) for 5 minutes. Frozen sections were left at room temperature for 10 minutes and then placed in cold (5°C) 4% paraformaldehyde (PFA, Sigma Aldrich, St Louis, USA) for 10 minutes and then they were allowed to dry completely. All slides were then circled with a PAP pen (Dako, Ely, UK) before being washed in phosphate-buffered saline (PBS, Life technologies, Paisley, UK).

2.6.2 Immunofluorescence

Sections were incubated with 0.1% trypsin (Gibco, Paisley, UK) at 37°C for 30 minutes; and washed twice in PBS for 5 minutes.

Slides were incubated in 0.6% H_20_2 and 0.1% triton X-100 in PBS for 10 minutes, washed twice in PBS (for 5 minutes) and incubated for 20 minutes in blocking buffer (10% goat serum, 0.1% bovine serum albumin, and 0.1% Tween-20 in PBS). After this the following primary antibodies were added and incubated for 90 minutes at room temperature see Table 2.4 for supplier and dilution. For CPD, 6-4PP, 8oxodG, 70 mM NaOH in 70% ethanol for 4 minutes was added first to denature the DNA). Sections were washed twice in PBS (5 minutes) and incubated with secondary antibodies: Alexa Fluor goat anti-mouse or goat anti-rabbit 488/555 (Invitrogen, Paisley, UK) at 1:200 for 30 minutes, counterstained with prolong gold antifade with DAPI (4',6-diamidino-2-phenylindole, Molecular Probes, Paisley, UK), cover-slipped, and stored away from light. See Table 2.5 for details on fluorescent probes used.

Location	Antibody	Host species	Species specificity	Clone	Supplier	Dilution
Nuclear	CPD	Mouse	Human	TDM2	Cosmobio, Japan	1:2000
	64-PP	Mouse	Human	64M2	Cosmo bio, Japan	1:300
	8oxodG	Mouse	Human	N45.1	JaICA, Shizuoka, Japan	5µg/ml (1:100)
	Isotype control	Mouse	Human	IgG1	Dako	At highest concentration (1:100)
	Ki67	Mouse	Human	Ab15580	Abcam, UK	1:100
	TRP1	Mouse	Human	Ab73873	Abcam, UK	1:100
	p53	Mouse	Human	DO1	Abcam, UK	1:500

	p63	Rabbit	Human	Ab53039	Abcam, UK	1:200
	CD68	Mouse	Human	Ab955	Abcam, UK	1:100
Extracellulr matrix	Keratin 1 (K1)	Rabbit	Human	Ab24643	Abcam, UK	1:100
	Keratin 14 (K14)	Rabbit	Human	Ab7800	Abcam, UK	1:100
	Keratin 10 (K10)	Rabbit	Human	Ab1421	Abcam, UK	1:100
	Involucrin	Rabbit	Human	Ab68	Abcam, UK	1:100
	MMP1	Rabbit	Human	Ab52631	Abcam, UK	1:100
	MMP12	Rabbit	Human	Ab66157	Abcam, UK	1:400
	MMP2	Rabbit	Human	Ab86607	Abcam, UK	1:300
	MMP9	Rabbit	Human	Ab58803	Abcam, UK	1:200
Secondary antibodies	Alexafluor 488	Goat	Mouse		Invitrogen, UK	1:200
	Alexafluor 488	Goat	Rabbit		Invitrogen, UK	1:200
	Alexafluor 555	Goat	Mouse		Invitrogen, UK	1:200
	Alexafluor 555	Goat	Rabbit		Invitrogen, UK	1:200

Table 2.4 Primary and secondary antibodies

Probe	**Excitation (nm)**	**Emission (nm)**	**Colour visualised**	**Experiment used in**
Fluorescein	494	521	Green	ISZ
BODIPY	505	515	Green	ISZ
AlexaFluor 488	495	519	Green	IF
AlexaFluor 555	555	565	Red	IF
DAPI	340-380	425	Blue	ISZ, IF

Table 2.5 Excitation and emmision spectra of fluorescence probes

2.6.3 Immunohistochemistry

Sections were immersed in Harris' Haematoxylin, (Sigma- Aldrich, UK) for 7 minutes then washed in running tap water for 1 minute. They were then dipped in 1% acid alcohol (1ml HCL, 100ml ethanol), 1% eosin, (Sigma-Aldrich, Dorset, UK), then washed with running tap water for 2-3 minutes. Slides were then rehydrated in graded ethanol. They were rehydrated in graded ETOH (50%, 70%, 90% and 100% for 2 minutes each) followed by xylene and then mounted using p-xylene-bis-pyridinium bromide (DPX, Sigma Aldrich, Dorset, UK).

2.7 *In situ* zymography (ISZ)

MMPs are released from nucleated cells as inactive proenzymes (Woessner, 1991). Although current primary antibodies pick up active forms, ISZ is useful for measuring the extent of MMP activity. This is done by applying a dye-quenched (DQ) substrate (collagen I, collagen IV, gelatin or elastin) to the section that has been heavily labelled with fluorescein isothiocyanate (FITC) so that its fluorescence is quenched (Mook *et al.*, 2003). After cleavage by the appropriate MMP, the degree of fluorescence released corresponds to activity. The added benefit with this is localisation of the activity in the skin. In this thesis, collagenolytic I and IV (DQI and DQ IV) activity and elastinolytic (DQ elastin) activity was assessed with the following protocol.

ISZ method

Frozen skin sections were prepared (see 2.6.1) and then incubated with 60µl substrate. This was made up of low gelling agarose (Sigma, Dorset, UK) (1g dissolved in 500ml PBS), with 4-6 drops of DAPI with antigold fade/1ml

(Invitrogen, Paisley, UK) and DQ™ collagen type 1, DQ™ collagen type 4 or DQ™ elastin (all from Molecular Probes, Paisley, UK)) at 1:10. Sections were allowed to set at 4°C for 1h and then placed at room temperature for ~18h in the dark. Substrate specificity is shown in Table 2.6.

Substrate	MMP specificity
Collagen I	MMP1, MMP2, MMP3, MMP9, MMP13
Collagen IV	MMP2, MMP7, MMP9, MMP12, MMP25
Elastin	MMP12, MMP7, MMP9
Gelatin	MMP2, MMP9

Table 2.6 In situ zymography MMP specificity taken from Yan and Blomme, 2003

2.8 Imaging analysis

Imaging was performed with a Zeiss Axiophot microscope (Harpenden, UK, Nikon DS-U2 camera (Kingston-upon-Thames, UK). Images were captured in 2560x1920 format at x20 magnification (and x1.25 eyepiece magnification). Contrast settings were kept constant and the gain and exposure times used for IF and ISZ are shown in Table 2.7. The SPS5 Leica confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) was also used to acquire images at x20 and x80 magnification at specific settings for p63, TRP1, Ki67, K1 and K14. These were set according to the unirradiated control and settings varied for each antigen.

Location	Antigen	Exposure (seconds)	Gain
Nuclear	CPD	20	x1.40
	64-PP	20	x1.40
	8oxodG	20-30s	x1.40-4.0[a]
	p53	4	x1.40
	DAPI	Variable (1-4)	x1.40
	Ki67	Confocal settings[b]	
	TRP1	Confocal settings	

	p63	Confocal settings	
Extracellular Matrix	**Keratin 1**	Confocal settings	
	Keratin 14	Confocal settings	
	Keratin 10	2	x1.40
	Involucrin	4	x4.00
	MMP1	4	x2.40
	MMP12	4	x2.40
	MMP2	2	x1.40
	MMP9	2	x1.40
	DQ ™ Collagen I	4	x4.00
	DQ ™ Collagen IV	4	x4.00
	DQ ™ Elastin	4	x2.40

Table 2.7 Fluorescence light microscopy capture settings for all antibodies used
[a] staining was attempted but could not be optimised
[b] confocal capture settings set for each unirradiated control

Epidermal and dermal quantification

This was performed with NIS elements BRv3 software package (Melville, NY, USA).

Nuclear staining

DAPI stained nuclei were gated and mean red intensity (AlexaFluor555) assessed within each nucleus, in at least 200 nuclei. Mean background intensity was calculated and intensity from each non irradiated control was subtracted from treatment sections to adjust for nonspecific nuclear staining. Settings for nuclei capture (circularity and diameter of nuclei) were constant, and nuclei ‘‘separated and smoothed’’ as necessary.

To assess staining as a function of depth in the epidermis, the 3MED sections from the thickest part of the epidermis from each of the 12 individuals were selected. The mean red intensity in each of the nuclei down to the basal epidermis was calculated and adjusted for average background staining in unirradiated controls. This was

plotted against the distance from the *stratum corneum*. For dermal staining, first the average basal epidermis thickness was calculated in µm (approximately 75µm). A total of 50 individual nuclei in the dermis from the 3MED sections from each of the 12 volunteers were gated and CPD/64PP staining assessed. Two calculations were done (once background unirradiated staining was removed) to show 1) staining in the dermis 2) combined staining in epidermis and dermis. Here, as there was an average of 10 nuclei in the epidermis across 75µm, average staining could be plotted across the whole skin.

Extracellular staining

Quantification was performed by gating the epidermis or dermis and then assessing the intensity of colour intensity within each region to give a numerical value.

2.9 Microarray

This section was performed by Milteyni Biotech, Bergisch, Gladbach, Germany.

Single colour hybridization on Agilent Whole Human Genome Oligo Microarrays (4x44K) and bioinformatics was performed (Milteyni Biotech, Bergisch Gladbach, Germany). 100ng of total RNA was used, converted to cDNA, and T7 RNA polymerase was used to simultaneously amplify and produce cyanine 3-labelled (Cy3) cRNA (Agilent Low Input Quick Amp Labeling Kit, Agilent technologies, Bershire, UK). Concentrations were >200ng/µl and this was measured with the ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). 1.65µg Cy3-labeled fragmented cRNA was hybridised (~17h, 65°C) to Agilent Whole Genome Oligo Microarray chips (4x44K) using the Agilent Gene Expression

Hybridization kit (Agilent technologies, Bershire, UK). Fluorescent signals were detected using the Agilent Microarray Scanner system and Agilent Feature Extraction Software (FES) was used to process the intensities. This software removes background, rejects outliers and calculates statistical confidences. Background corrected intensity values were normalized between arrays using quantile normalization (Bolstad *et al.*, 2003) where sample values were divided by their median. Log_2 transformed intensity values were used for subsequent statistical analysis. Rosetta resolver gene expression data analysis system, (Rosetta Biosoftware) was used to compare single intensity profile in a ratio experiment (i.e. control versus sample experiment). The microarray data are deposited at NCBI GEO with accession number GSE45493. The data were then processed using specific software programmes (Genego Metacore v7 and the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 see section 2.11.

2.10 Polymerase Chain Reaction (PCR)

2.10.1 Reverse transcription polymerase chain reaction (RTPCR)

RTPCR was performed following the manufacturer's protocol (high capacity cDNA reverse transcription kit, Applied Biosystems, Paisley, UK). Master mix was prepared (see Table 2.8) in a 1:1 ratio with total RNA (90-188ng/µl). The mix was incubated at 25°C for 10 minutes, 37°C for 120 minutes and 85ºC for 5 minutes on the G-Storm GS4 Thermal cycler (Labtech International Ltd). cDNA was stored at -20ºC.

Component	Volume (□l) per well
10xRT Buffer	2
25xdNTP Mix	0.8
10xRT Random Primers	2
Multiscribe RT	1
Nuclease-free H20	4.2
Total	10
Total RNA	10

Table 2.8 The reverse transcription mix

2.10.2 Real time quantitative PCR (RTqPCR)

Real time quantitative PCR (RTqPCR) assesses mRNA fold change compared to control unirradiated skin using the $2^{-\Delta\Delta CT}$ method (Yuan *et al.*, 2006). The TaqMan® Gene Expression assay (Applied Biosystems, Paisley, UK), uses FAM™ (6-carboxyfluorescein) labelling and is quenched by dihydrocyclopyrroleindole (MGB) whilst the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, Paisley UK), is VIC™ (*undisclosed formulation from Applied Biosystems*) labelled and quenched with MGB. This enables duplex experiments (i.e. probe and housekeeping gene in each well) for analysis, which were made up as shown in table 2.9 using cDNA made in section 2.10.1. The selection of these genes was based on the microarray data as described in Chapter 5.

Agent	Quantity per well (µl)
Probe	1
GAPDH	1
Master mix	10
Distilled water	6-7.5 (depending on the amount of cDNA used)
cDNA	0.5-2 (depending on probe used)

Table 2.9 RTPCR master mix

RTPCR was performed using the Applied Biosystems 7900HT fast real time PCR system (50ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute). Reporter (FAM/VIC) fluorescence was captured using SDS 2.3. The genes validated are shown in table 2.10.

Pathway:	**Gene symbol**	**Assay ID**
Inflammation	IL1ß	Hs00153133_m1
	IL6	Hs00174103_m1
	IL8	Hs00174103_m1
	TNF	Hs00174128_m1
Extracellular matrix	MMP1	Hs00899658_m1
	MMP3	Hs00968305_m1
	MMP9	Hs00234579_m1
	MMP10	Hs00233987_m1
	MMP12	Hs00899668_m1
	MMP13	Hs00233992_m1
Immunosuppression	CD83	Hs00188486_m1
	IL10	Hs00961619_m1
Antioxidant activity	HO-1	Hs01110250_m1
	FOSL1	Hs04187685_m1
	PTGS2	Hs00153133_m1
	NRF2	Hs00232352_m1
Apoptosis	TNFAIP3	Hs00234713_m1
	BCL2	Hs00608023_m1
	MDM2	Hs00242813_m1
	TP53	Hs01034249_m1

Table 2.10 Genes validated with RTPCR using TaqMan ® DNA probes

2.11 Statistical Approaches

For microarray data, bioinformatics analysis was based on normalized Log_2 intensities using R/Bioconductor and software packages (http://www.R-project.org; http://www.bioconductor.org). ANOVA with repeated measurements followed by Tukey post-hoc tests was used to test for expression differences among the following

groups: UVA1 time course, UVA1 6h vs UVA1 24h (50J/cm^2 doses), UVA1 vs UVB at 6h, UVA1 vs UVB at 24h. All p values were adjusted for multiple testing (Benjamini and Hochberg, 1995). Differentially expressed genes were considered if both the ANOVA and Tukey post-hoc test p-values were ≤ 0.05 (adj. p-value or false discovery rate (FDR) ≤ 0.3) and the expression difference was at least two fold.

Functional analysis of candidate genes was done using DAVID v 6.7 (Dennis *et al.*, 2003) to identify biological clusters and GeneGo Metacore v7 to identify key pathways. Briefly, gene ratio lists for pooled individuals (fold change ≥ 2, $p \leq 0.05$) from the 6h and 24h biopsies were uploaded to the software program, and in DAVID these gene lists orientate into biological clusters or Gene Ontology (GO) groups. With GeneGo, upregulated gene lists are processed and this generates pathway maps, networks, processes and diseases and the p values given show the statistical likelihood of a sequence of genes occuring with a particular pathway due to chance. For mRNA and protein analysis, all graphs were generated using Graphpad Prism v6 or Microsoft Excel for Mac 2011 for statistical tests including linear regression analysis and unpaired/paired t tests, or Mann Whitney/Wilcoxon signed-rank test depending on the normality of the sample values.

Chapter 3 : UVA1 induces CPD but not 6-4PP *in vivo* in human epidermis and dermis

Published papers:

Tewari A, Sarkany R.P. and Young A.R.

UVA1 induces cyclobutane pyrimidine dimers but not 6-4 photoproducts in human skin *in vivo*.

Journal of Investigative Dermatology 2012;132(2):394-400

Tewari A, Lahmann C, Sarkany R, Bergemann J, Young AR.

Human erythema and matrix metalloproteinase-1 mRNA induction, in vivo, share an action spectrum which suggests common chromophores.

Photochemical and Photobiological Sciences 2012;11(1):216-23.

Tewari A, Grage MM, Harrison GI, Sarkany R, Young AR.

UVA1 is skin deep: molecular and clinical implications.

Photochemical and Photobiological Sciences 2013;12(1):95-103

Abstract

UVB readily induces cyclobutane pyrimidine dimers (CPD), mainly thymine dimers (TT), and pyrimidine (64) pyrimidone photoproducts (64PP) in DNA and has recently been shown to occur following UVA exposure without concomitant production of 6-4PP in explant skin. In this chapter, the induction of TT and 64PP in human skin *in vivo* by broadband UVA1 has been investigated, and compared with erythemal doses of monochromatic UVB (300 nm). Our data show that UVA1 induces TT in the epidermis of healthy volunteers *in vivo* and did not form 64PP, whilst UVB induced both types of photoproduct, suggesting different mechanisms of action for induction. UVB induces 3-4 times more epidermal TT than UVA1 for the same level of erythema. UVA1 induced TT increased with epidermal depth with predominance at the rapidly dividing basal epidermis whilst the reverse pattern was seen with UVB and a predominance at the upper epidermis. This pattern is also seen in the dermis with a continued decrease of UVB induced CPD with depth and an increase with UVA1 induced CPD with depth. UVA1 and UVB induced TT are repaired at a similar rate across the whole epidermis, but repair is much slower for UVA1 induced CPD when kinetics at the basal epidermis are examined only. Our conclusions focus on how UVA1 may be more carcinogenic than has previously been thought which has important implications for public health education and the use of tanning lamps.

3.1 Introduction

It is well established that UVA forms CPD in cultured cells and human whole explant skin, and that these lesions are more frequent than oxidatively generated lesions such as 8oxodGua (Douki *et al.,* 2003; Ravanat *et al.,* 2001). A series of studies have demonstrated that UVA causes damage to DNA (Basu-Modak and Tyrrell, 1993; Kielbassa *et al.*, 1997; Tyrrell and Pidoux, 1989), protein and lipids via indirect mechanisms, primarily mediated by singlet oxygen (Morita *et al.,* 1997). Direct absorption and damage to DNA has also been proposed (Jiang *et al.,* 2009; Mouret *et al.,* 2010) for the formation of CPD, where dAdT may absorb UVA1 directly, particularly at 354nm (of note second erythema peak at 360nm (Anders *et al.,* 1995) see Introduction 1.4.1.1).

As both UVB and UVA predominantly form TT dimers (Mouret *et al.*, 2006), we used a monoclonal antibody directed against the TT dimer to assess CPD in human skin *in vivo* after erythemally equivalent doses of UVB (300nm) and UVA1 (340-400nm), as well as a monoclonal antibody against 64PP. Erythema was used as the dose determinant because it is a key response to UVR damage and is widely used to define UVR exposure. Furthermore, human action spectroscopy has specifically implicated TT formation as a trigger for erythema (Young *et al.,* 1998a) particularly at 300nm (Freeman *et al.,* 1989), as well as other biological effects (Walker and Young, 2007) in the skin. In addition to induction assessed by a dose response study, we assessed the repair of photoproducts (time course study) with UVA1 and UVB *in vivo*, because it has been reported that TT repair is slower *ex vivo* after UVA1 than UVB, which is relevant to the formation of mutations (Mouret *et al.*,

2006).

3.2 Materials and Methods

Materials and methods are described in Chapter 2.

Volunteer Demographics

Table 2.1 gives the volunteer demographics for the time course study (6 volunteers) and dose response study (6 volunteers) and the mean MED for all 12 skin type I/II participants was 25.8 ± 4.6 mJ/cm^2 (UVB) and 45.8 ± 9.4 J cm^2 (UVA1).

3.3 Results

3.3.1 Staining of control tissue

It was not possible to stain and assess all slides in a single batch. The reproducibility of staining and image analysis was measured for both the dose response and time course studies by staining the same positive control slides (3MED) for UVA1 and UVB with each experimental run with background unirradiated control sample intensity removed. Figure 3.1A and B show the small standard deviation for 3MED UVA1 and UVB sections with each staining run, confirming that any variation in immunostaining was unlikely to be due to operator bias.

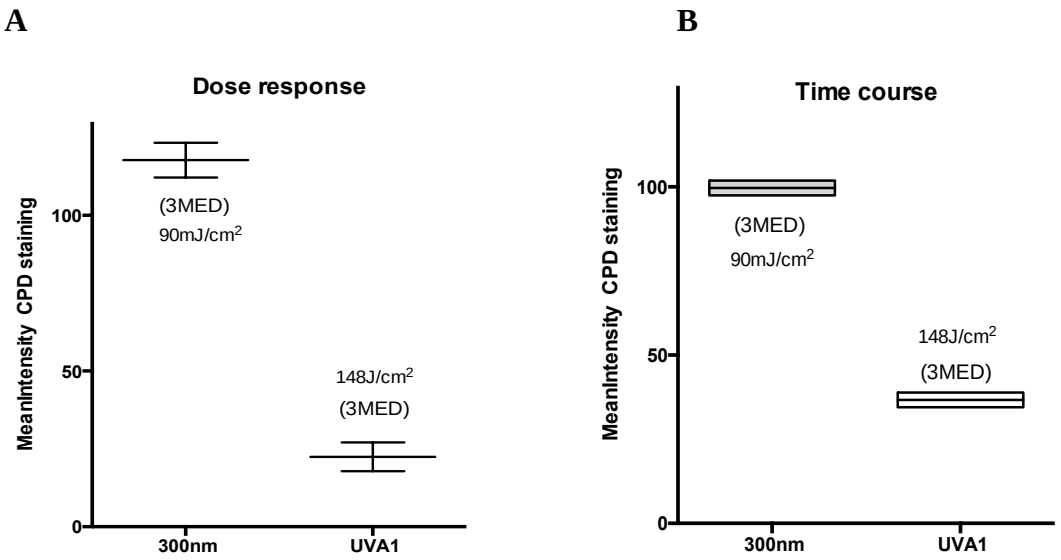


Figure 3.1 Staining of control tissue when biopsies were taken immediately after exposure

Error bars represent minimum and maximum values. Mean intensity refers to amount of fluorescence; the greater the amount of CPD the greater the intensity.

3.3.2 Erythema

The erythema index (EI) was calculated by converting the incremental exposures for MED assessment to factors of the notional MED.

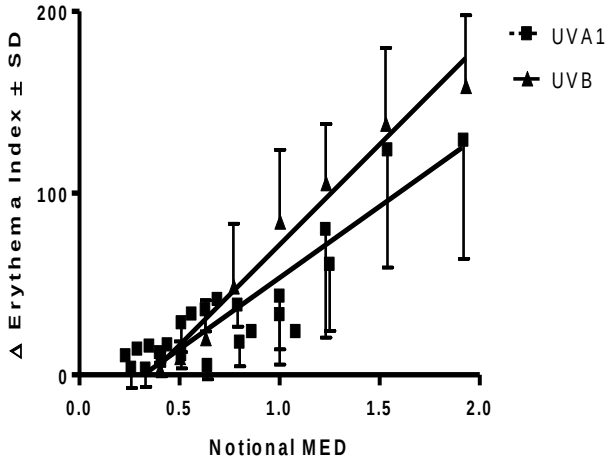


Figure 3.2 Erythema dose response based on notional MED
The data show that the slopes for both UVA1 and UVB are highly significant (UVB $p = 0.001$, UVA1 $p = 0.0006$) and there was no difference between the slopes ($p = 0.21$), i.e. there are comparable dose responses for UVB and UVA1.

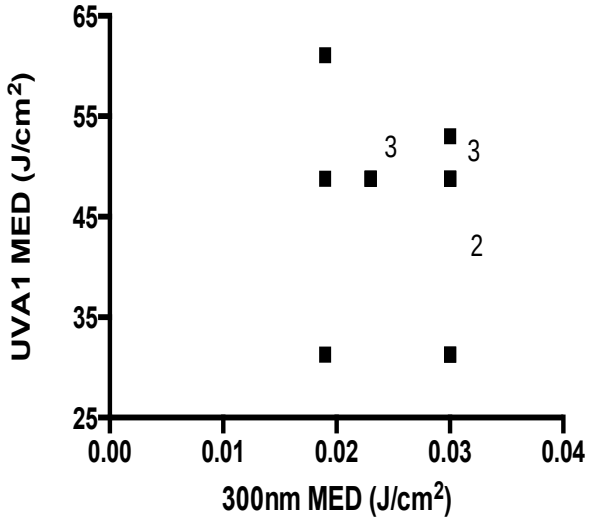


Figure 3.3 UVA1 vs UVB erythema
There was no correlation between a given individual's UVB and UVA1 MED (n = 12: note numbers on the figure correspond to the number of volunteers with the same MED).

3.4 CPD and 64PP staining

Representative examples of CPD and 64PP staining for the dose response study and the time course study are shown in Figure 3.4 and Figure 3.5 respectively.

3.4.1 CPD and 64PP quantification

Dose response and time course staining quantification is shown in Figure 3.6 and Figure 3.7. The dose-response study showed that, for a given multiple of the notional MED, UVA1 produced fewer TT than UVB. The slope for UVB is 3.4 fold steeper than for UVA1 ($p = 0.001$). 64PP are induced by UVB in a dose-dependent manner but no 64PP are induced with UVA1 at any dose *in vivo.*

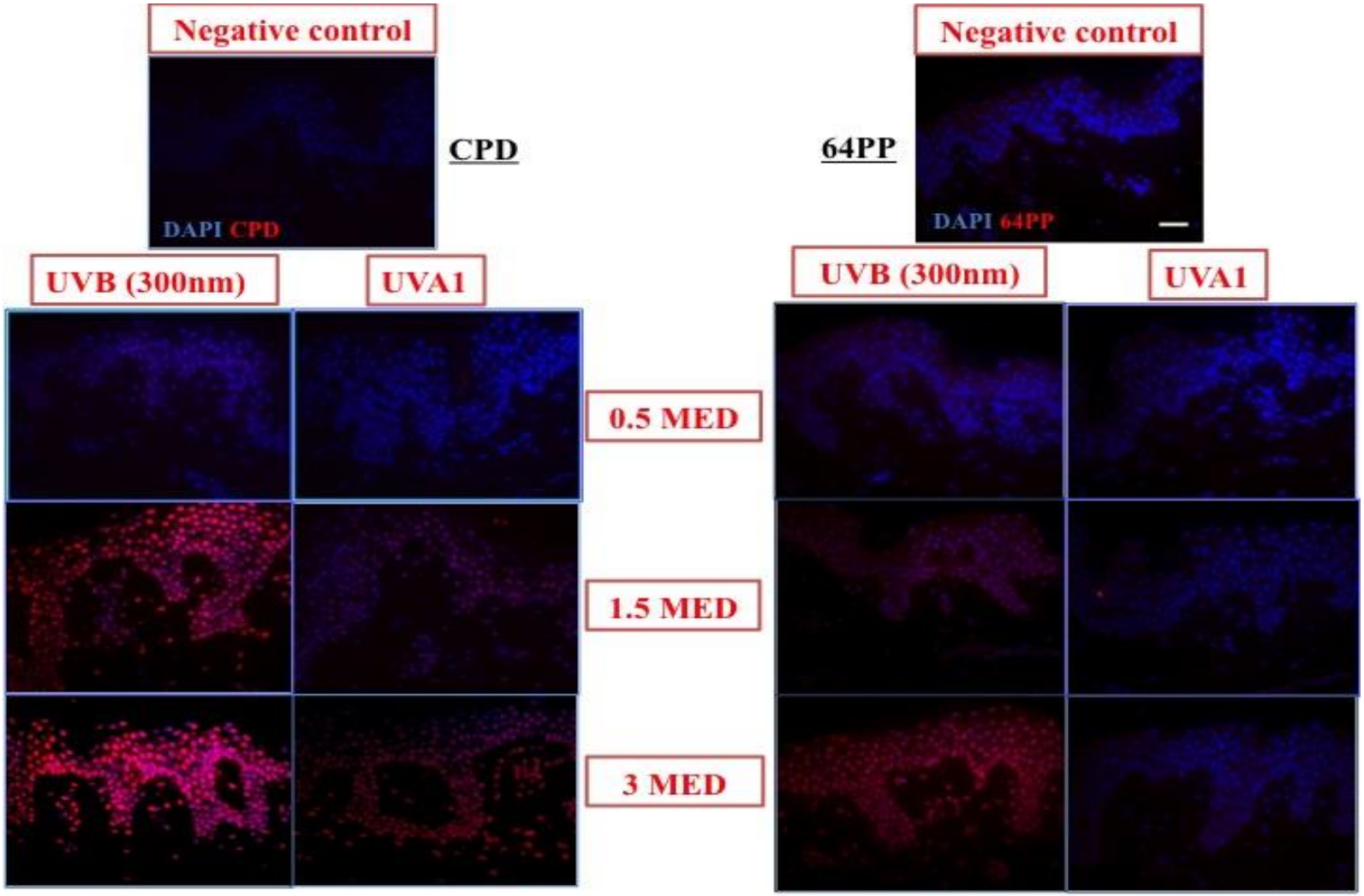


Figure 3.4 Dose response staining (scale bar=50μm)
CPD staining (red) and DAPI or nuclear staining (blue) showing increased CPD at greater MED for UVA1 and UVB.

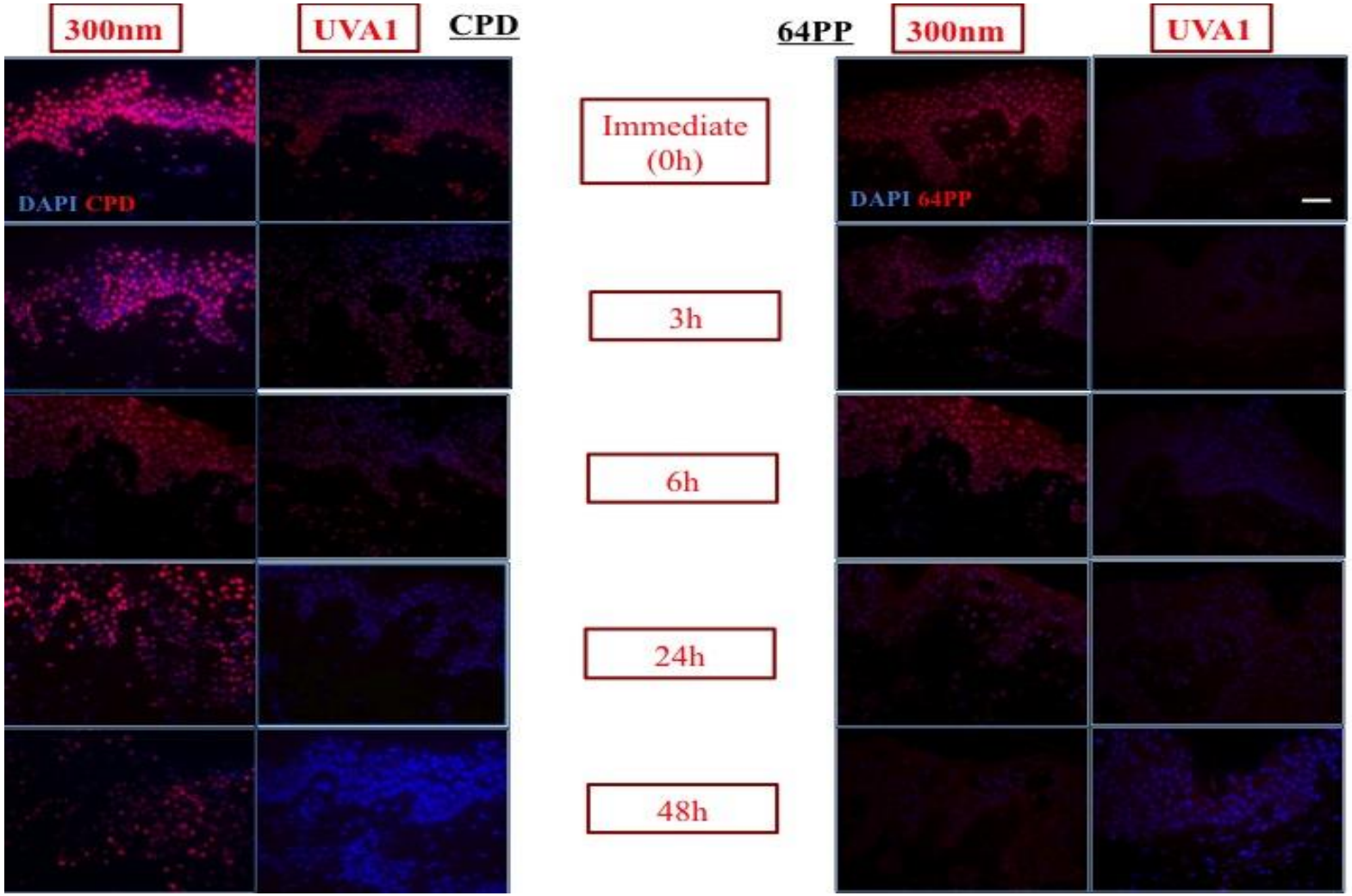


Figure 3.5 Time course staining (scale bar=50μm)
CPD staining (red) and DAPI or nuclear staining (blue) showing increased CPD at greater MED for UVA1 and UVB.

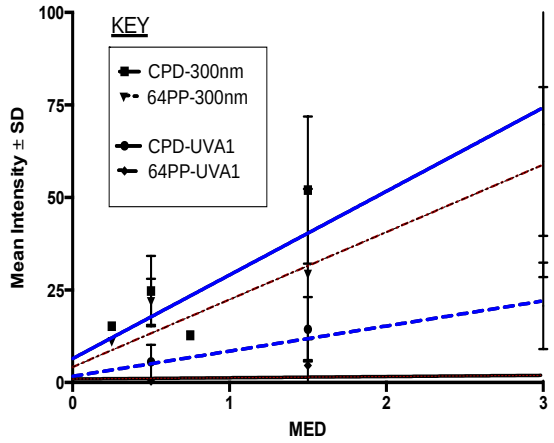


Figure 3.6 Dose response quantification for UVB and UVA1 induced CPD and 64PP at 0h.

A B

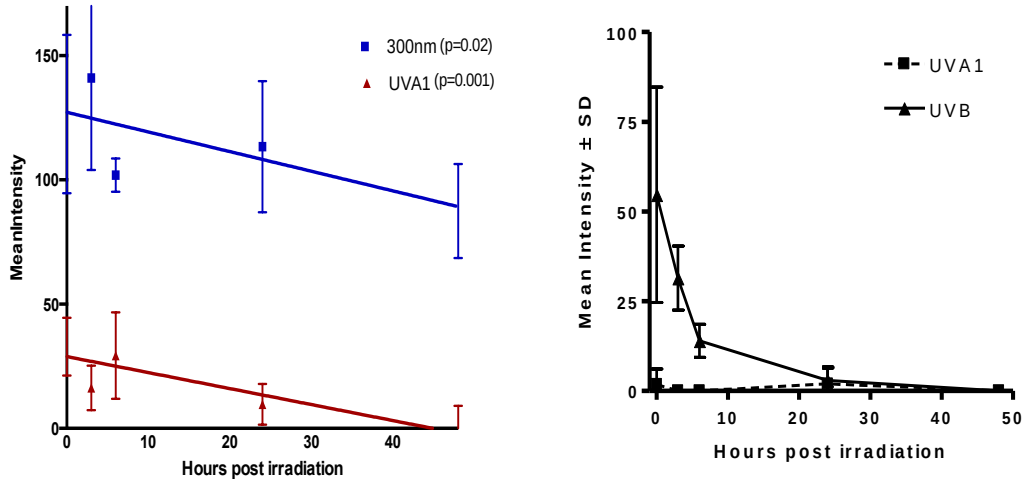


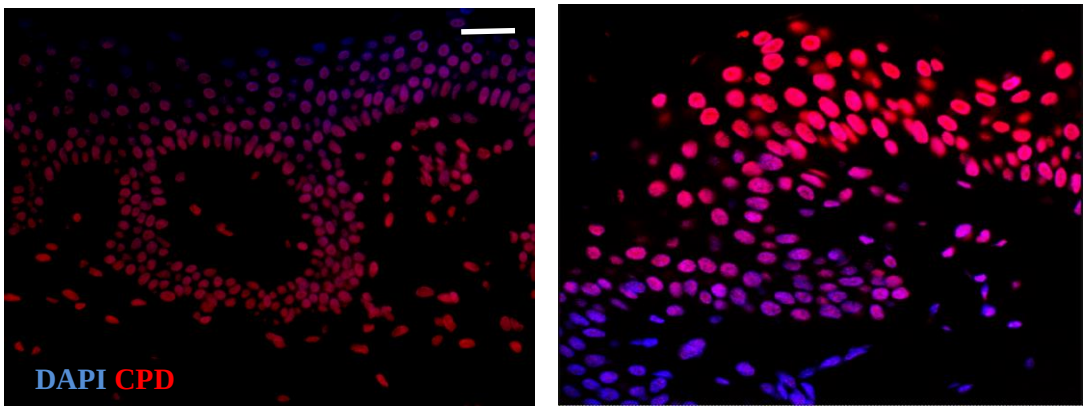
Figure 3.7 Time course for repair kinetics of (A) CPD and (B) 64PP in whole epidermis

Examining the time course repair kinetics for CPD (Figure 3.7A), the slopes for the loss of UVB and UVA1 induced CPD with time are not significantly different ($p = 0.71$). By 48h, the majority of UVA1 induced CPD has disappeared. However, UVB induced CPD are still present. The comparable repair rate for CPD induced by both spectra suggests that the same host repair machinery is used. Our data are different from published *ex vivo* studies (Mouret *et al.*, 2006) that suggest that UVB induced CPD is repaired faster than those induced by UVA1. Repair of UVB

induced 64PP is fast and complete by 24h, as previously reported *in vivo* (Bykov *et al.*, 1999; Young *et al.*, 1996) for UVB using SSR.

3.4.2 Distribution of CPD staining in epidermis

A B



C

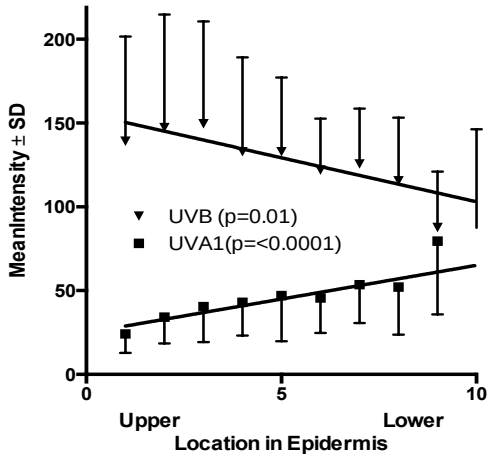


Figure 3.8 Distribution of CPD in epidermis at 3MED immediately post-UVR
Example of UVA1 (A) and UVB (B) CPD staining across the epidermis in 3MED sections and (C) quantification per nuclei through the epidermis. Scale bar = 50μm.

UVA1 causes more intense staining in the lower epidermis, whereas UVB leads to more intense upper epidermal staining. The relationship between staining intensity

and epidermal depth was quantified by linear regression analysis to a depth of 10 nuclei (Figure 3.8C). With UVB there was significant (p = 0.01) reduction in CPD staining with increased epidermal depth. In contrast, for UVA1, there was a significant (p = 0.001) increase in staining with increased depth. This pattern of staining is similar at lower doses. With 1.5MED and 0.5MED the patterns are the same, but the statistical significance is variable which is probably related to the fact that actual intensity values are smaller, and at 1.5MED there is a statistically significant attenuation of UVB induced CPD (p = 0.006) across the epidermis but not for UVA1 CPD (p = 31). Interestingly at 0.5MED there is a significant increase in CPD intensity across the epidermis with UVA1 (p = 0.001) but no effect with UVB (p = 0.25).

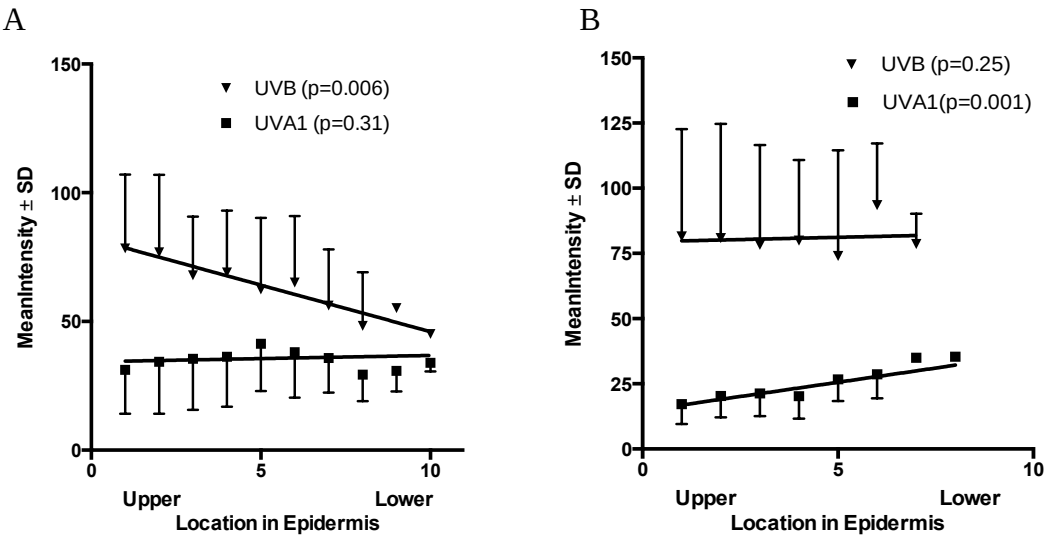


Figure 3.9 CPD staining across the epidermis at different doses (A) 1.5MED (B) 0.5MED

3.4.3 Distribution of CPD staining in dermis

Using the dose response and time course tissue, CPD staining was calculated in the dermis for UVA1 and UVB at 0.5MED, 1.5MED and 3MED (Figure 3.10). As

expected, there is a dose dependent increase in CPD for UVA1 (slope p = 0.009) and UVB (slope p < 0.0001) according to notional MED but maximal damage with UVB is only 2.5 fold greater that with UVA1, compared to 3-4 fold difference in the epidermis. Figure 3.11A shows the degree of CPD damage is independent of depth (p = 0.53 for slope) for UVA1, whereas there is a highly significant (p = 0.0001 for slope) reduction in CPD with depth with UVB. Figure 3.12 shows that repair of dermal CPD is slow for UVB. The slope for UVB is not significant (p = 0.62) but is significant for UVA1 (p = 0.03).

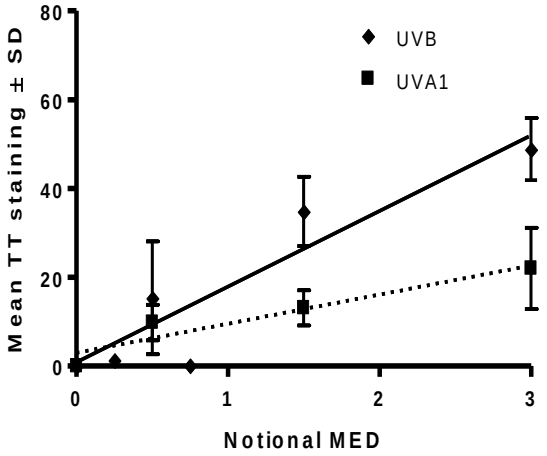


Figure 3.10 CPD dose response in the dermis

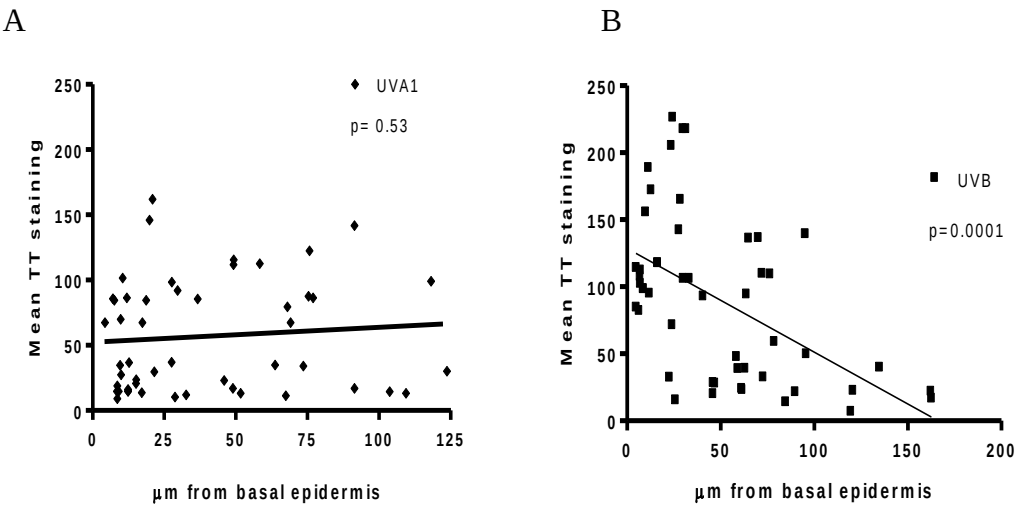


Figure 3.11 CPD damage according to depth in the dermis after 3MED
(A) UVA1 (B) UVB CPD

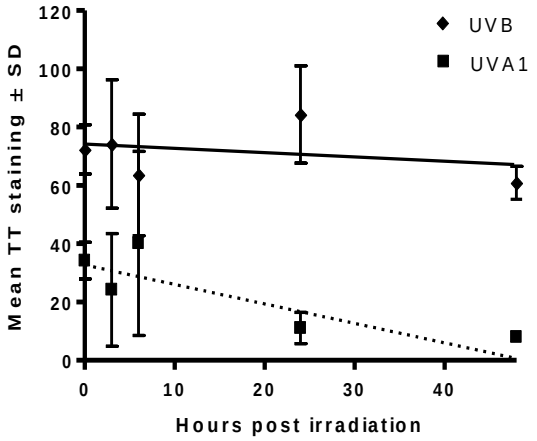


Figure 3.12 CPD repair over 48h in the dermis

3.4.4 Combining epidermal and dermal data

Data for epidermal and dermal CPD and 64PP formed by UVA1 and UVB have been combined. Figure 3.13A shows a statistically significant attenuation of UVB induced CPD through the whole skin ($p < 0.0001$) and a statistically significant increase in UVA1 induced CPD ($p = 0.006$). The regression lines intercept at 150µm, which implies that beyond this depth, UVA1 induces more CPD than UVB.

There is attenuation with UVB induced 64PP (Figure 3.13B) with depth, but UVA1 does not produce any 64PP at any level. Figure 3.14 shows attenuation of both UVB induced CPD and 64PP with increasing depth with no significant difference between the slopes.

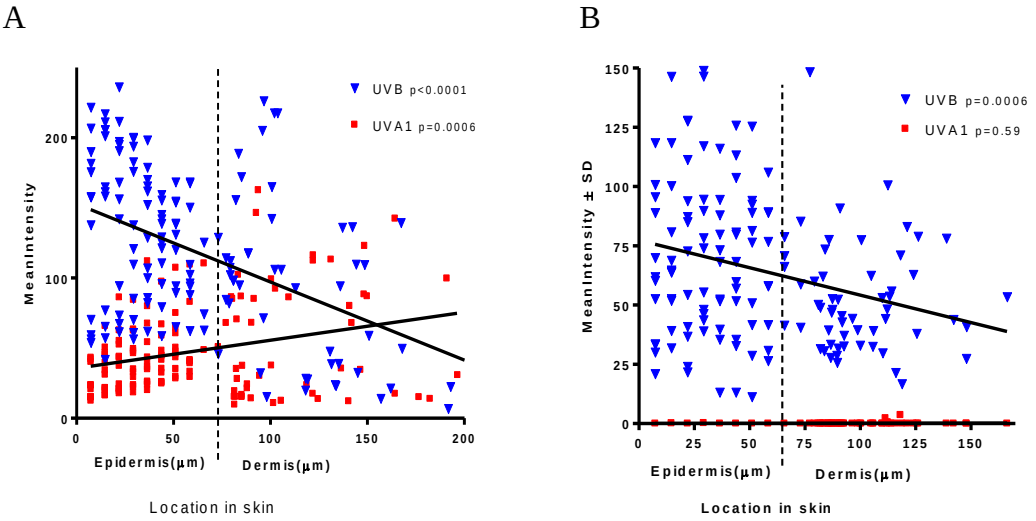


Figure 3.13 Combined epidermal and dermal photoproducts for UVA1 and UVB (A) CPD (B) 64PP

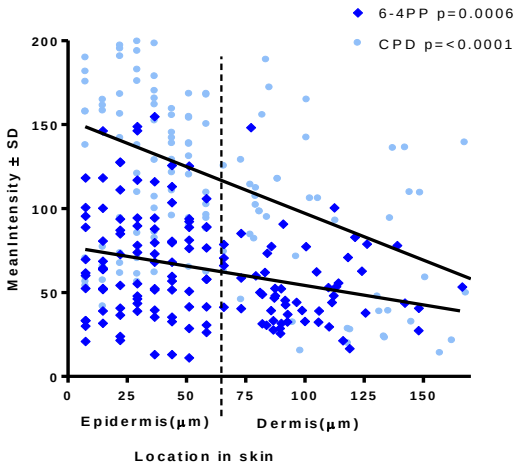
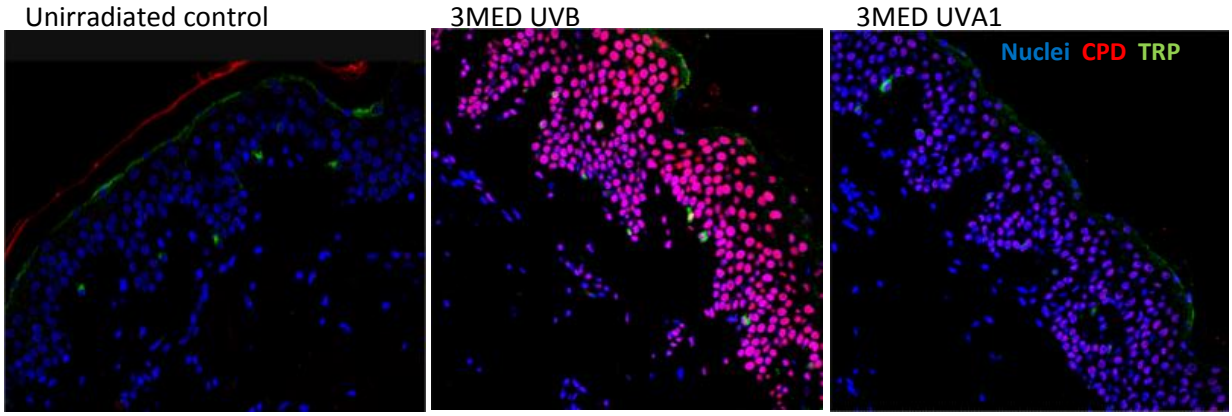


Figure 3.14 UVB induced CPD and 64PP in the skin

3.4.5 UVA1 CPD are not repaired at the basal epidermis

The intensity of CPD staining present at the basal layer of the epidermis (melanocytes, identified by TRP1) and basal keratinocytes was examined over 48h and plotted below.

A



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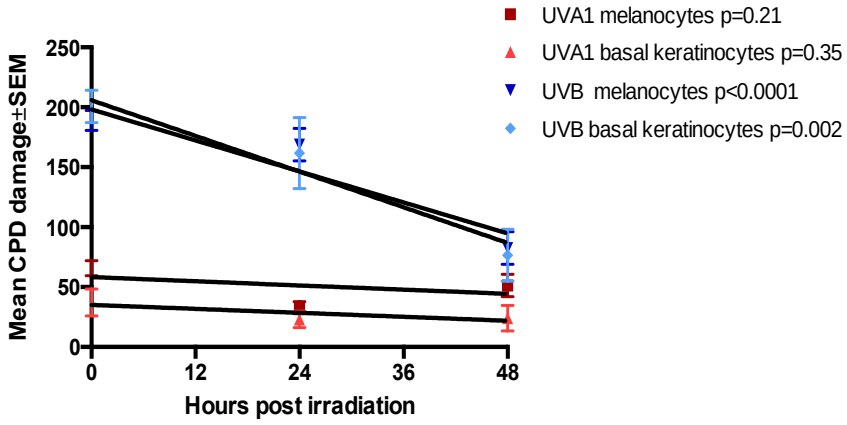


Figure 3.15 CPD staining in melanocytes and basal epidermal keratinocytes
(A) example of CPD and TRP1 staining in unirradiated control, 3MED UVB and 3MED UVA1 (B) quantification of staining with linear regression p values

Unexpectedly UVA1 and UVB repair kinetics are very different at the basal epidermis (Figure 3.15C) and although UVA1 produces 3-4 fold fewer CPD, these

generally do not appear to be repaired (linear regression of slope p = 0.21 for melanocytes and p = 0.35 for basal keratinocytes) whereas UVB induced CPDs are repaired quickly in melanocytes (p = 0.002) and basal keratinocytes (p =<0.0001) over 48h. Mechanisms are examined in Chapter 4.

3.5 Discussion

3.5.1 UVA1 induces CPD, but not 64PP in the epidermis and dermis

The TT dimer is the most common type of DNA lesion formed by UVB and UVA (Douki *et al.*, 2003; Mouret *et al.*, 2006). UVB forms TT>TC>CT>CC and UVA forms TT>TC>CT but not CC dimers (Mouret *et al.*, 2006) in explant skin. The data in this chapter show that at doses which give equivalent erythema, UVA1 readily induces TT but not 64PP in human skin *in vivo* albeit 3-4 times fewer than UVB induced TT. Our maximum doses for UVB (300nm) and UVA1 were 90mJ/cm^2 and 148J/cm^2 respectively compared with 20mJ/cm^2 and 200J/cm^2, used in a large comparative study on *ex vivo* skin (Mouret *et al.*, 2006), where here the UVB source was 312 nm. 20mJ/cm^2 was sub-erythemal as an MED for skin type I/II at 312nm has been shown to be 200–300mJ/cm^2 in skin type I/II individuals (Palmer *et al.*, 2006). A further study irradiating biopsy samples *ex vivo* found similar levels of TT damage after 1MED of UVB and UVA however their source contained considerable amounts of UVA2 (Mouret *et al.*, 2011b).

3.5.2 CPD repair

An *ex vivo* study has found that UVA1 induced TT are repaired less well than those induced by UVB (Mouret *et al.*, 2006) but doses were not biologically relevant. Repair kinetics data in this chapter show *in vivo,* when the epidermis is examined as

a whole, CPD are repaired at the same rate whether they are induced by UVA1 or UVB (Figure 3.7), and as UVA1 produced fewer CPD than UVB, repair was almost complete at 48h post 3MED UVA1. Thus it appears that the degree of initial DNA damage from an acute exposure determines the amount of damage that remains a day or two later. However, *in vitro* studies suggest that the rate of CPD repair from a single exposure may decrease with higher doses, possibly resulting from damage to the DNA repair machinery (Courdavault *et al.*, 2004; Greinert *et al.*, 2000). The situation appears to be different with repeated daily sub-erythemal exposures with SSR, which results in the accumulation of TT (Young *et al.*, 2007), although this varies with skin type. Repeated sub-erythemal exposure enhances TT repair in skin types III/IV but not in I/II (Sheehan *et al.*, 2002). In contrast, mice exposed to chronic low dose UVB show decreased CPD repair following a challenge dose of UVR suggesting that an accumulation of TT had a detrimental effect on repair (Mitchell *et al.*, 1999). Figure 3.11 and Figure 3.12 show there is no evidence of repair of UVB induced CPD in the dermis whilst UVA1 induced TT repair is slow which has also been shown by others (Bykov *et al.*, 1999; Young *et al.*, 1996) using an SSR source.

3.5.3 Location of CPD in the skin

It is intriguing that UVB predominantly induced TT in the superficial epidermis, whereas UVA1 induced TT were more prominent in the lower epidermis (Figure 3.8). A similar depth effect has been found with p53 protein staining at 24h (Campbell *et al.*, 1993a) post 2MED UVB (300 ± 5 nm) and UVA (350 ± 30 nm) and also at 1MED but not 2MED doses of UVA1 (Burren *et al.*, 1998). Overall, skin

chromophores absorb primarily in the UVB region. UVA is known to make up a greater percentage of UVR wavelengths that reach the basal layer of the epidermis (Miller *et al.*, 1998), and in explant skin (Bruls *et al.*, 1984), 12% of the UVB reaches basal Caucasian epidermis compared with >25% UVA. As well as transmittance and scatter, back scattering results in remittance, which provides additional opportunity for chromophore absorption during the return pathway. Dermal remittance increases between 300 and 400nm (Anderson and Parrish, 1981), thus it is possible that the higher number of UVA1 induced TT seen in the basal layer is due to dermal back scatter (e.g., from collagen and fibroblasts), as well as epidermal forward scatter (Bruls *et al.*, 1984). Irrespective of any mutagenic potential for TT, our data suggest that UVA1 preferentially targets the stem cell containing basal layer.

Mechanism of CPD production by UVA1

UVA is poorly absorbed by DNA (Sutherland and Griffin, 1981) and absorption beyond 360nm is questionable. Approximately 3 orders of magnitude more energy from UVA1 than UVB is needed to give equivalent erythema (Diffey *et al.*, 1987), as also shown with our erythema data: a UVB MED in skin type I/II is 30mJ/cm^2 and a UVA1 MED is 48.8J/cm^2. Direct evidence that DNA is a chromophore for erythema has been obtained from animal studies (Ley, 1985), however the similarity of the TT and erythema action spectra *in vivo* especially at 300-340nm range (UVB and UVA2) suggests that erythema is a marker of CPD mediated through a common chromophore: via DNA (Freeman *et al.*, 1989; Young *et al.*, 1998a). This has also been recently confirmed in a further *in vivo* study (Mouret *et al.*, 2011b) using UVB

and UVA sources where equivalent levels of erythema in skin types II and IV are associated with similar levels of TT. Figure 3.3 shows a lack of correlation between an individual's UVB and UVA1 MED suggesting that UVA1 induced erythema likely occurs from at least 2 chromophores one of which is DNA and the other is still yet to be identified.

Induction of CPD and 64PP

With direct absorption of UVB, T (and occasionally C) enters an excited singlet state that then undergoes intersystem crossing to an excited triplet state (Cadet and Douki, 2011). This energy is transferred to a ground state pyrimidine, causes a formal (2+2) cycloaddition between the C5-C6 double bonds of two pyrimidines and the formation of a CPD (see Introduction). Pyrimidines remaining in an excited singlet state will form a 64PP. Varying energy levels achieved by pyrimidines probably explains the differing distributions of CPD and 64PP. CPD are formed by triplet energy transfer after direct UVB absorption by thymine or cytosine (Douki, 2013), but can also be formed by UVA1. As 64PP are not produced by UVA1, singlet state energy states are likely a UVB mediated phenomenon.

There are currently two schools of thought for TT induction by UVA; either a triplet energy transfer mechanism, or an emerging view that they are formed via weak direct absorption of UVA by DNA. Formation of dimers by UVA and UVB is summarized in Figure 3.16.

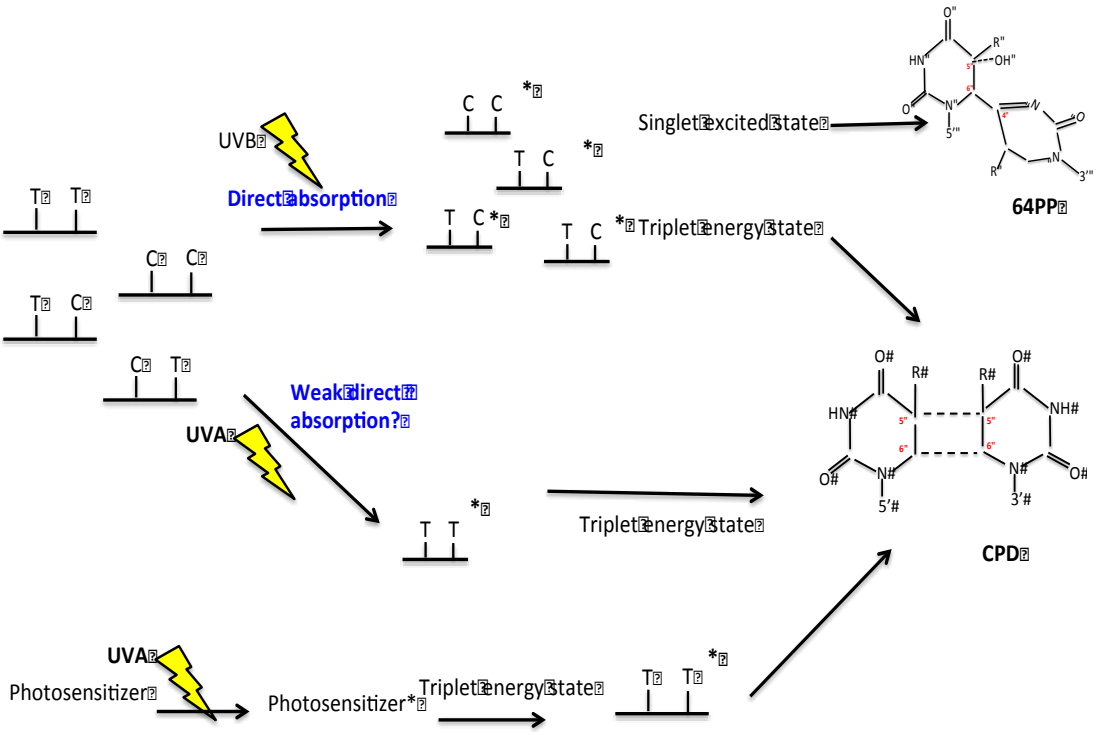


Figure 3.16 Mechanism of CPD and 64PP induction by UVA1 and UVB

UVA1 induced CPD formation:

Indirect mechanism

UVA1 induced CPD (Cadet *et al.*, 2009) form via triplet energy transfer. Briefly, photons are absorbed by a photosensitizer that enters an excited singlet excited state, and then undergoes intersystem crossing to achieve an excited triplet state. This triplet state persists for slightly longer, and enables energy to be transferred to thymine predominantly (as it has the lowest triplet energy and can easily absorb this energy and initiate new changes). This causes the formation of TT CPD (90% of UVA CPD) (Mouret *et al.*, 2006). Support for an as yet unidentified endogenous photosensitizer comes from work with exogenous photosensitisers (Sauvaigo *et al.*, 2001; Traynor and Gibbs, 1999), which can initiate the formation of CPD in the

presence of UVA. These include iomefloxacin (Sauvaigo *et al.*, 2001) acetophone (Douki *et al.*, 2003), carprofen (Robinson *et al.*, 2010) and fluoroquinolone (Makinen *et al.*, 1997) at UVA doses as low as 5J/cm^2. They act as the excited photosensitizer and indirectly transfer UVA photons to DNA. Interestingly, a recent population study showed an increased risk of BCC and SCC by 50% and 80% respectively in those patients taking photosensitizing drugs such as psoralens, tetracyclines, fluoroquinolones, amiodarone and phenothiazines (Karagas *et al.*, 2007), possibly due to the increased induction of CPD with UVA exposure.

Direct mechanism

A few reports discuss the formation of CPD in naked acellular DNA after UVA irradiation (Jiang *et al.*, 2009; Kuluncsics *et al.*, 1999; Quaite *et al.*, 1992; Schuch *et al.*, 2009), particularly in the absence of a photosensitizer. Genomic DNA has been shown to absorb weakly at UVA wavelengths (Sutherland and Griffin, 1981), but this early work may be misleading as cell cultures used had a predominance of G:C base pairs which absorbs UVA particularly poorly. In cultures composed solely of A:T (which absorb UVA well) (Mouret *et al.*, 2010), recent experiments show (Mouret *et al.*, 2010) that direct UVA (320 – 400nm) absorption by duplex dA:T oligomers produce TT dimers at similar proportions to TT produced in cells, suggesting that this phenomenon may be occurring through direct absorption in keratinocytes.

Generally, photosensitization by other (non-DNA) skin constituents occurs with UVA, and may explain why there is less TT induction for equivalent levels of

erythema. UVA1 erythema has been shown to be oxygen dependent (Auletta *et al.*, 1986) likely mediated through ROS (Tyrrell, 2000), whilst UVB erythema is oxygen independent.

3.5.4 UVA mutations in skin cancer

The role of UVA in skin cancer is a growing concern for several reasons. First, UVA was found to be mutagenic *in vitro* (Drobetsky *et al.*, 1995; Kappes *et al.*, 2006; Rochette *et al.*, 2003), tumorogenic in mice (de Gruijl and Van der Leun, 1994; de Laat *et al.*, 1997; van Kranen *et al.*, 1997) and proposed by some authors to be involved in the induction of MM (Setlow, 1974; Setlow *et al.*, 1993; Setlow *et al.*, 1989) although not all have agreed with these findings (De Fabo *et al.*, 2004). UVA forms melanoma through formation of reactive melanin radicals (Wood *et al.*, 2006) which has been further confirmed in recent mice studies (Noonan *et al.*, 2012) interestingly by a group who previously refuted melanoma induction by UVA (De Fabo *et al.*, 2004). UVA induced melanoma occurs in pigmented but not in albino mice (lacking melanin) following production of significant quantities of 8oxodG (Noonan *et al.*, 2012), which fundamentally secures a significant role of the G-C→T-A transversion mutation in melanoma. Interestingly UVB did not produce 8oxodG in this study. These observations are supported by work that shows that melanocytes are particularly sensitive to UVA damage (Mitchell and Fernandez, 2012; Mouret *et al.*, 2011a). UVA probably acts in a mechanistically different way in both SCC and melanoma and although C→T mutations are the predominant mutations in p53 in SCC (Benjamin *et al.*, 2008; Brash *et al.*, 1991) and in a melanoma cell line (Pleasance *et al.*, 2010), the G→A transversion is the second

common mutation in melanoma (Pleasance *et al.*, 2010).

3.5.5 Summary

Environmental relevance of UVA1 doses

On a mid summer's day in the south of France, the maximum ambient daily UVR erythemal dose is around 22 MED and the maximum UVA dose is around 137 J/cm^2 (Fourtanier *et al.*, 2012), whilst on average, an indoor worker in Europe is exposed to 1,500 J/cm^2 UVA per year (Diffey, 1996). In our study we used single UVA1 doses (24.4, 73.2. 146.4J/cm^2 equivalent to 0.5MED, 1.5MED and 3MED), which formed TT dimers. It is known that frequent users of UVA-rich sunlamps increase their annual UVA exposure. In countries such as Norway, the UVA irradiances of tanning beds are 3–3.5 times that of summer solar exposure (Nilsen *et al.*, 2008) meaning that regular tanning bed users would quadruple their annual UVA exposures (Miller *et al.*, 1998). Cumulative exposure to UVA1 also occurs during treatment of certain dermatological skin disorders such as morphea (Andres *et al.*, 2010; Su *et al.*, 2011) where on average patients receive cumulative doses of approximately 900 J/cm^2 UVA1 (50 J/cm^2, 3 times a week for 6–8 weeks) in one year, whilst for treatment for other conditions such as atopic eczema of the hands or cutaneous T-cell lymphoma, single doses of up to 100 J/cm^2 UVA1 (Kerr *et al.*, 2012b) mean that cumulative yearly exposures can be up to 1800J/cm^2.

Impact on Public Health

UVA1 is found in very large doses in tanning lamps as it is less erythemogenic and hence thought to promote tanning without the potentially painful side effects of

sunburn (Mutzhas *et al.*, 1981). It is important to note that approximately 80% of tanning lamp emission is UVA1, 18% UVA2 and 2% UVB (*personal communication from Patrick Tierney*) and UVB is more effective than UVA in initiating tanning. Thus, commercial tanning is likely a contribution of both UVB/UVA2 delayed tanning and UVA1 immediate photooxidation of melanin. In terms of carcinogenic effects the exact contribution is unknown and skin biopsies from chronic tanners have not been examined for molecular changes.

The trend to use commercial sunlamps for cosmetic pigmentation has increased over the past few decades, with 71% of users being women (Swerdlow and Weinstock, 1998) and 50% under the age of 29 (Coelho and Hearing, 2010). Melanomas are the most prevalent of all cancers in 25-29 year old females, and melanoma incidence is higher in young women compared to men (Morris *et al.*, 2009).

The tanning industry promotes sunbed use as risk free, suggesting that it is a safe method for vitamin D synthesis yet there is overwhelming evidence that UVA contributes to skin cancers. A UVB tan provides a small sun protection factor of 2 (Sheehan *et al.*, 2002), supported by chemical assays that show that UVB increases melanin production (Wolber *et al.*, 2008). UVA causes photooxidation of melanin precursors at lower doses than for neomelanogenesis (see Introduction) and there is generally no sun protection factor achievable with UVA. The recent decision of the International Agency for Research on Cancer (IARC) to classify artificial UVR devices as carcinogens (El Ghissassi *et al.*, 2009) further emphasizes the carcinogenic risk associated with UVA, importantly with reference to sunlamps. The IARC has shown that there is a 75% increase in melanoma risk (IARC, 2007) where the first exposure to a sunbed was before the age of 35. This is also confirmed

by a more substantial meta-analysis (Boniol *et al.*, 2012) identifying an 87% higher risk of developing melanoma compared to non sunbed users if use began before 35 years. Significant risk also occurs with SCC induction, with a 67% increase in likelihood compared to non-sunbed users if exposure began under 25 years (Wehner *et al.*, 2012). A recent study in the UK showed that by the age of 55 years the risk of SCC induction from sunbed exposure between the ages of 20-35 years is increased by 90% (Tierney *et al.*, 2015).

Although there is EU legislation to control sunbed exposures, a recent British study showed that in practice this is unregulated: 9 out of 10 sunlamps emit above legislation limits for irradiance (Tierney *et al.*, 2013), and more importantly these lamps were not manned by health professionals. Melanoma is triggered by intermittent sunburning UVR exposures. An estimated 18-55% of commercial sunlamp users in Europe and North America have reported sunburns (Demko *et al.*, 2003), with a higher proportion occurring in women (Geller *et al.*, 2002). This risk needs to be emphasized to young adults who are at greatest risk.

Currently, in some US states, minors (under 18) are not allowed to use sunbeds. In practice, the situation is more complex because a nationwide study showed that some parents are complicit in allowing their children to engage in tanning. This is because compared to other high-risk behaviours that have potentially serious acute consequences, UVR exposure risks are viewed as less serious and the long-term consequences are poorly understood (Pichon *et al.*, 2009). In the UK there is a law (Sunbeds Regulation Act 2010) that prohibits sunbed use by those under 18

(*http://www.legislation.gov.uk/ukpga/2010/20/section/2).* The demographics of sunbed use after this legalisation would be useful to look at as sunbed use is extremely frequent in the 11-17 year age group in the UK (Thomson *et al.*, 2010).

The work in this chapter has relevance for public health strategies: for consolidating legislation of sunbed use as well as stressing the importance of good broadband photoprotection which is already increasingly recognized (Osterwalder *et al.*, 2014). At present, young fair skinned women, who have the greatest risk for melanoma, are heavily targeted by the sunbed industry and younger generations tend to ignore warnings in favour of brown, sun-damaged skin, thus extending their potentially carcinogenic exposure to UVR. More CPD are formed the deeper in the skin below 150μm after UVA1. CPD contributes to MMP1 formation and increased MMP1 levels in the dermis might help explain the efficacy of UVA1 in diseases such as scleroderma (Kreuter *et al.*, 2006). UVA1 phototherapy is expensive (~£30,000 per machine as opposed to narrowband UVB (TL01) that costs ~£3000-4000) but has recently been set up in Leeds and is also available in London and Dundee.

Chapter 4 UVA1 and UVB have different effects on determinants of apoptosis and DNA repair

Abstract

UVA1 induced CPD *in vivo* are predominantly formed in the basal epidermis and their repair is slow over 48h. This layer is crucial for epidermal proliferation because it is home to epidermal stem cells. In Chapter 3 we show a lack of repair of UVA1 induced CPD in the basal epidermis. To investigate the slow repair, a series of other differentially expressed epidermal markers known to be sensitive to UVR (p63, p53, as well as epidermal differentiation markers (involucrin, Keratin (K)1, K10, K14) and proliferation marker (Ki67) in the individuals from the DNA repair time course study (6 individuals) and from the Protein validation gene study (3 participants) were assessed.

UVA1 induced expression of p63 over 48h whilst UVB decreased its expression. Only UVB induced p53 expression and SBC (apoptosis) formation.

CPD recognition, DNA repair and apoptosis are p53 driven processes and both UVB and UVA1 induce CPD. The basal epidermis is relatively more sensitive to UVA1 than UVB although in total more CPD are produced by UVB across the whole epidermis. The overexpression of p63 at the basal epidermis by UVA1 is likely to suppress p53 in this layer and affect the downstream p53 driven processes causing a reduction in cellular arrest and apoptosis and may explain why UVA1 CPD are not recognised and repaired over a 48h period. Although 3-4 fold fewer, this might mean that UVA1 induced CPD are possibly more mutagenic than UVB CPD. If this is the case, it has implications for the safety of UVA1 phototherapy, tanning lamps and the use of sunscreens that protect only against UVB.

4.1 Introduction

The mutagenic and carcinogenic potential of UVA1 *in vivo* at erythemally equivalent doses of UVA1 (3MED (~148J/cm^2) and UVB (3MED ~90mJ/cm^2) was discussed in Chapter 3. Although these doses were high, they are biologically relevant for UVA1 phototherapy (Kerr *et al.*, 2012a), cumulative tanning lamp exposure and long-term outdoor exposure. This chapter focuses on other markers that are UVR sensitive, such as p53 and its recently cloned homologue p63, as well as markers of keratinocyte differentiation in order to understand some of the cellular processes in the skin in response to UVA1 particularly the lack of repair of UVA1 induced CPD at the basal epidermis.

Tumour protein p53

Activation of the p53 tumour suppress gene (TSG) and its protein occurs in response to cellular stress including DNA damage, oncogenic stimulation and hypoxia. This triggers a G1/S cell cycle arrest and transcriptional activation of NER genes (p48XPE and XPC) to enable CPD removal and NER, before onset of scheduled DNA synthesis and mitosis (Smith and Fornace, 1997). p53 also induces apoptosis (SBC formation) through Bcl2 (Burns *et al.*, 2002) family members (BAX, PUMA) and an upregulation of the death receptor FAS through a caspase associated pathway. This process eliminates DNA damaged keratinocytes, that are potential progenitors of malignant tumour cells (see Figure 4.1) adapted from (Smith and Fornace, 1997)).

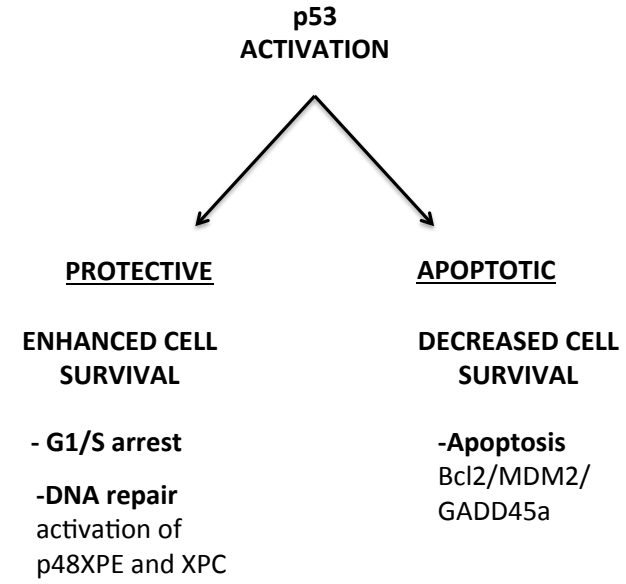


Figure 4.1 Activation of p53 and its downstream consequences
p53 has dual roles; protective via G1/S arrest and DNA repair and induction of apoptosis via Bc2/MDMD2/GADD45a.

The role of p53 in UVR induced apoptosis is clear from mouse studies (Jiang *et al.*, 1999; Ziegler *et al.*, 1994) in which UV irradiation of p53 wild type mice induced the formation of SBC whilst p53 null mice were resistant to such apoptosis and accumulated CPD. The p53 gene is mutated in more than 90% of human SCC and about 50% of human BCC (Brash *et al.*, 1991; Ziegler *et al.*, 1993) as explained in Chapter 3, and combined analysis of skin cancer mutations (SCC and BCC) showed that 90% of the mutations were G:C→A:T in XP patients (Dumaz *et al.*, 1993; Kraemer, 1997). p53 mutations prevent cellular arrest and repair, and through a lack of p53 function, these transition mutations are incorporated into further rounds of replication with tumour initiation and promotion resulting in SCC and BCC. UVR induced C→T mutations have been found in 80% of p53 mutations in actinic keratosis (Ziegler *et al.*, 1993) and 63% of p53 mutations in Bowen's disease (Campbell *et al.*, 1993b). In sporadic human cancer cases, mutations in p53 occur in approximately half of cases (Kraemer, 1997).

p63

p63 is a member of the p53 gene family (Westfall and Pietenpol, 2004) and exists in multiple isoforms with varying functions. All isoforms contain an N terminal (either transactivating (TA) or truncated (ΔN)), a C terminal (α, β, γ, δ, ε) and a p53 response element, which enables interaction with p53. Two main isoforms: TAp63 and ΔNp63 exist. Most work has focused on the ΔNp63 isoform as TAp63 negative mice do not display any overt abnormalities. ΔNp63 is most abundantly expressed in human epidermal basal layer (Vanbokhoven *et al.*, 2011).

The crucial role of p63 (ΔNp63) in the formation of the epidermis is demonstrated from studies in knockout mice. p63 deficient mice have no epidermis, aberrant squamous epithelia (cervix and urothelium) and are also devoid of epithelial appendages such as hair follicles (Parsa *et al.*, 1999; Yang *et al.*, 1999). p63 knockout mice also fail to commit to an epidermal lineage, which results in an epidermis with a single cell layer (Mills *et al.*, 1999), and no expression of markers of epidermal development and differentiation. As p63 promotes terminal differentiation, data regarding upregulation of keratin markers in response to UVB and UVA1 is also presented in this chapter.

Terminal differentiation of epidermal keratinocytes

Keratinocytes undergo a distinct pattern of proliferation and differentiation (Fuchs, 1990), and upwards migration resulting in the formation and maintenance of the stratum corneum (consisting of dead corneocytes and secreted lipids). This is known

as terminal differentiation, and is essential for the skin's function as a protective barrier against entry of environmental chemicals and microbes and protection against dehydration (Blanpain and Fuchs, 2006).

Classically the epidermis comprises 2 cell compartments: one consisting of undifferentiated proliferative cells occupying the basal layer and the other consisting of differentiated post mitotic cells occupying the suprabasal layers (Lavker and Sun, 1982, 2000; Regnier *et al.*, 1986). Work has shown that the basal epidermis is actually a heterogeneous cell layer, and as well as the undifferentiated stem cells (epidermal stem cells) there are also differentiated post mitotic cells that are normally seen in the suprabasal layers (Regnier *et al.*, 1986).

The stem cell layer continually produces keratinocytes, and also secretes and assembles an ECM that constitutes much of the underlying basement membrane as well as laminins that hold the epidermis together. The major structural proteins of the epidermis are the keratins, and the expression of different keratin types is related to the stage of differentiation. The basal layer is typified by the expression of K5 and K14. When cells are committed they move upwards, switch off integrin and laminin expression and execute a terminal differentiation programme where the intermediate suprabasal layers express K1 and K10, and filaggrin and loricrin are produced higher up in the granular layer. As keratinocytes commit to terminal differentiation, p63 expression is lost (Parsa *et al.*, 1999; Westfall and Pietenpol, 2004). p63 expression is needed to sustain K1, K10, loricrin and desmoglein expression (Truong *et al.*, 2006) and in ΔNp63 null mice the spinous layer of the epithelium fails to develop properly as demonstrated by a delay in expression of K1 (Koster and Roop, 2008). p63 is needed for epidermal commitment and stratification.

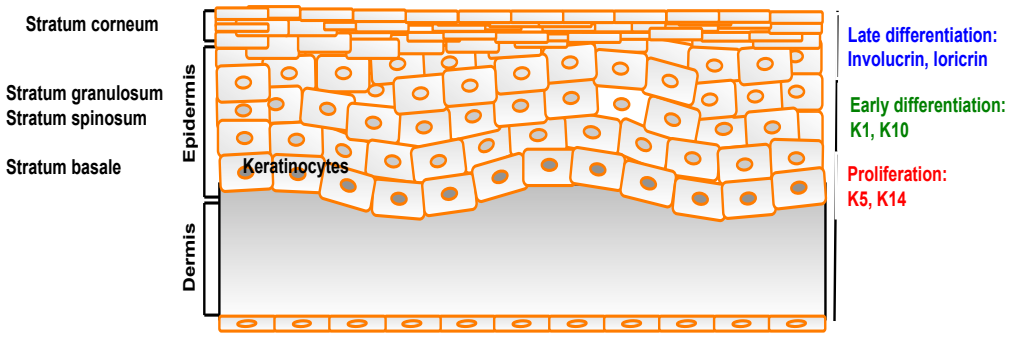


Figure 4.2 Markers found in the epidermis according to differentiation stage

Stem cells can be activated by stress events such as wounding when tissue regeneration is needed. The slow-cycling attribute of stem cells conserves the cell's proliferative potential until needed and minimizes DNA replication related errors. The division of stem cells also gives rise to a further stem cell known as a transit amplifying (TA) cell (1:1 ratio) and the TA cell is rapidly proliferating, during which it can increase the number of rounds of DNA replication. Once proliferative potential is exhausted TA cells undergo terminal differentiation (Lavker and Sun, 2000).

p53 is inducible by UVR and can also be inhibited by the ΔNp63a isoform (Yang *et al.*, 1998) as in cell lines the percentage of cell death induced by the ΔNp63 was considerably less than those cells expressing p53. p63 is also UVR responsive (Liefer *et al.*, 2000): its down regulation appears to be important for UVB induced apoptosis (Liefer *et al.*, 2000). Transgenic mice constitutively overexpressing ΔNp63a exhibit a 40-45% decrease in UVB induced epidermal apoptotic cells compared with non-transgenic littermates suggesting important physiological interactions.

4.2 Materials and methods

Volunteer demographics

Taken from the Time course DNA damage study (6 individuals) and from the Protein validation gene study validation experiments (3 participants) (see Table 4.1).

Staining

The types of staining performed are shown in the table below.

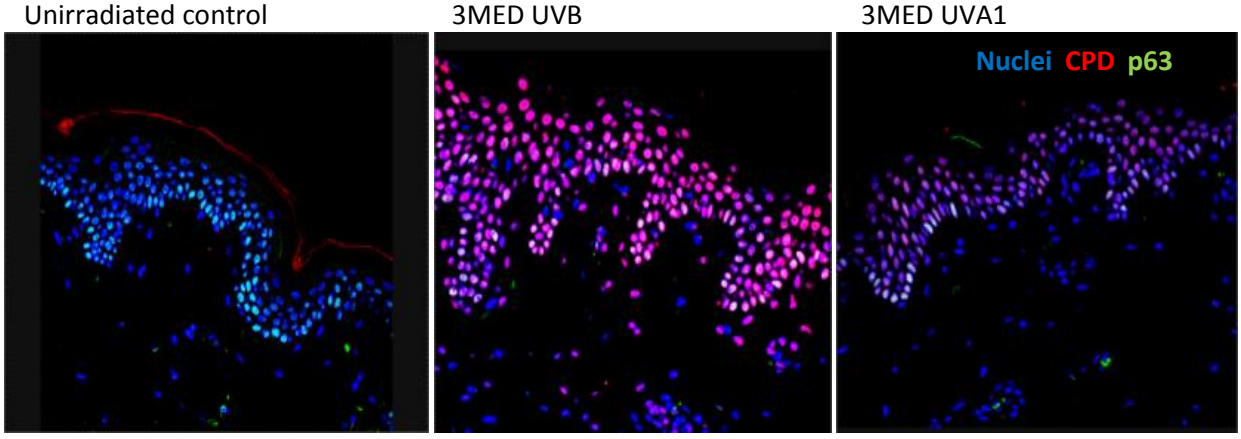
Study group	Staining
3MED: Paraffin embedded sections from the DNA damage time course study (n=6)	p63, CPD, H&E Epidermal differentiation: involucrin, K1, K10, K14 Proliferation: Ki67
1MED: Frozen sections from the gene protein validation study (n=3)	p53

Table 4.1 Types of immunostaining on treated tissue
MED refers to 30mJ/cm^2 for UVB and 50J/cm^2 for UVA1

4.3 Results

4.3.1 UVA1 induced upregulation of p63 expression in basal epidermis

A



B

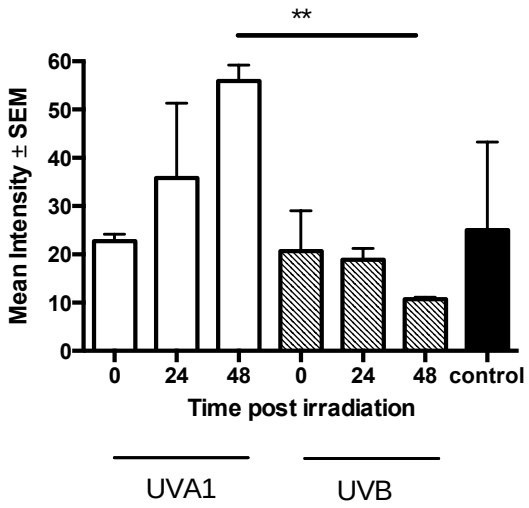
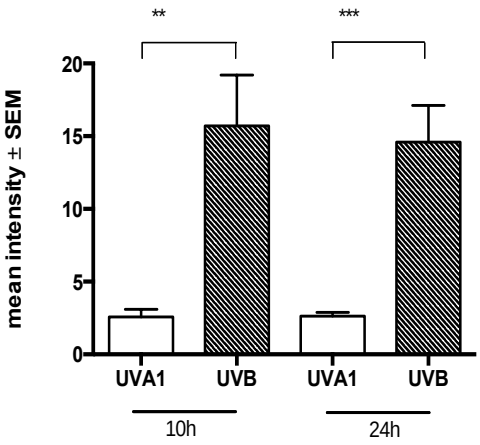


Figure 4.3 Spectra specific effects on basal epidermis
(A) Example of CPD and p63 co-staining in skin sections at 24h. Note the superimposed blue (DAPI), green (p63) gives a deep aqua colour seen in the unirradiated control. The third superposition of red (CPD) makes the colour more purple in the 3MED UVB and UVA1 slides (B) Quantification of p63 at the basal epidermis: increase in p63 expression following 3MED UVA1 at the basal epidermis compared to 3MED UVB (** p=0.001)

In the basal epidermis, 3MED UVA1 induced a statistically significant increase in p63 expression (p =0.047) and 3MED UVB induced a decrease in p63 expression (p=0.041). (see Figure 4.3B). Statistics on the intensity of p63 staining at 48h showed a significant difference between UVA1 and UVB (p=0.001).

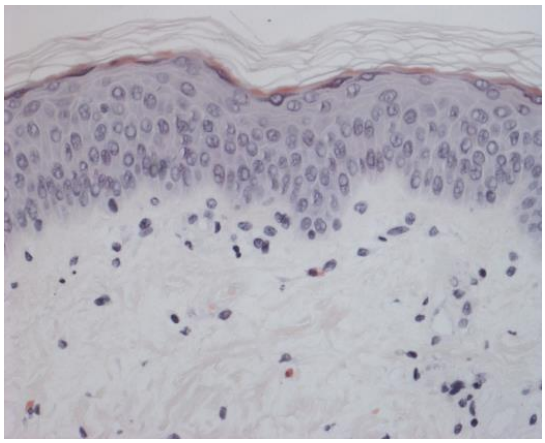
4.3.2 UVA1 does not induce p53 protein expression or sunburn cell formation

A

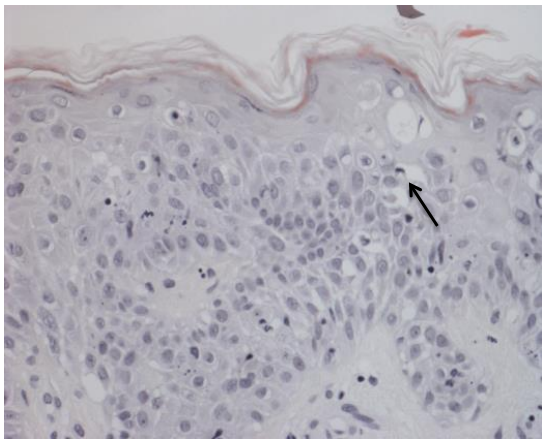


B

3MED UVA1 at 24h



3MED UVB at 24h



C

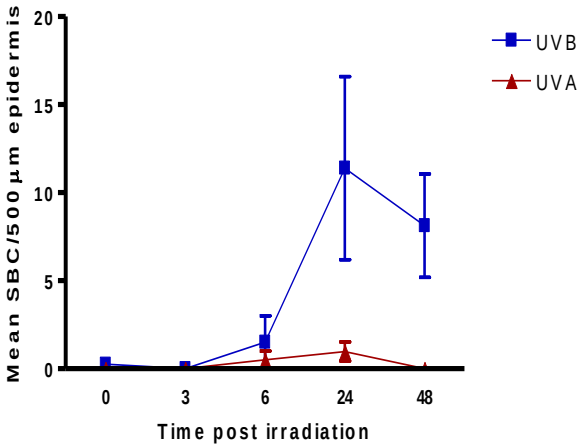


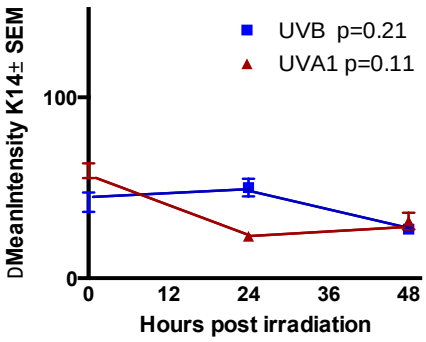
Figure 4.4 p53 and sunburn cell formation
(A) p53 expression after 1MED UVA1 and 1MED UVB at 10h (** p=0.004) and at 24h (*** p=0.0008), 3MED doses (B) examples of H&E staining: arrow points to a typical SBC (C) mean number of SBC per 500µm epidermis after 3MED. Error bars refer to standard error (SEM).

There was minimal p53 expression with 1MED UVA1 compared to 1 MED UVB at 10h (p=0.004) and 24h (p=0.0008) see Figure 4.4A. There was no SBC formation with UVA1.

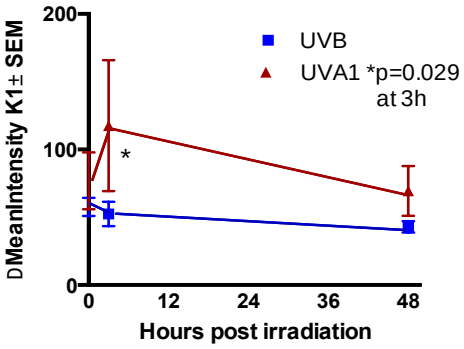
4.3.3 Terminal differentiation and proliferation

In terms of differentiation markers, K14, K1, K10 and involucrin were examined after 3MED UVA1 and UVB for any time dependent changes up to 48h (Figure 4.5A-D). Only K1 expression (found in suprabasal layers) showed a source specific difference at 3h between UVA1 and UVB, and appeared to be upregulated by UVA1 ($p=0.029$). Ki67 staining was found throughout the epidermis, and UVB caused increased nuclear proliferation (Ki67) at 3h compared to UVA1 (Figure 4.5E). Epidermal thickness was also measured from the H&E sections and was found to be notably thicker after UVB than after UVA1 (Figure 4.5F).

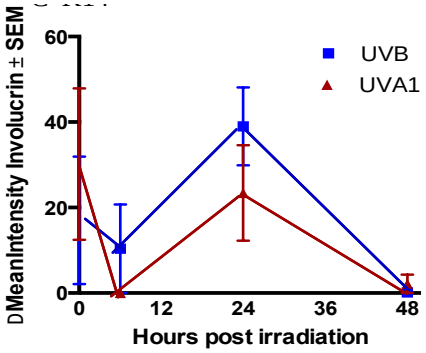
A K14



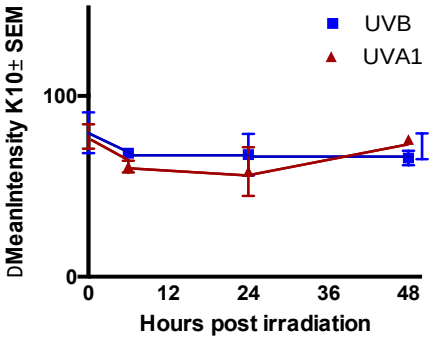
B K1



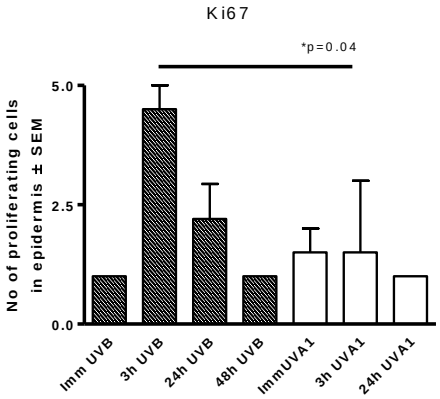
C Involucrin



D K10



E Ki67



F Epidermal thickness

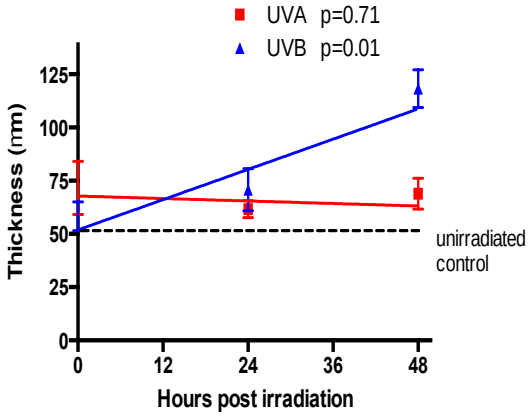


Figure 4.5 Spectral specific morphological changes to the epidermis
(A)-(D) Changes to 4 epidermal markers in response to 3MED UVB and UVA1 over a period of 48h: increase in UVA1 induced K1 expression at 3h compared to UVB (*p=0.03) (E) increase in cellular proliferation at 3h after UVB compared to 3MED UVA1 (*p=0.02). Background control staining has been removed from all slides (F) changes to epidermal thickness

4.4 Discussion

UVA1	**UVB**
Increased basal epidermal p63 at 48h	Decreased basal epidermal p63 at 48h
No/limited p53 production	p53 production
No SBC	SBC formation
K1 formation (3h)	No increased K1 formation
No change in K10, K14, involucrin	No change in K10, K14, involucrin
No increased Ki67	Ki67 expression (3h)
No increase in epidermal thickness over 48h (p=0.71)	Increased epidermal thickness over 48h (p=0.01)

Table 4.2 Summary findings in this chapter
UVA1 and UVB induced cellular changes over a 48h period in response to 3MED UVA1 and UVB.

p63 and terminal differentiation

The data show that a single 3MED UVA1 exposure induces basal epidermal p63 expression (ΔNp63α isoform) that reaches maximal levels at 48h (p=0.04), whereas UVB decreases p63 expression over the same period of time (p=0.041). At 48h there is a significant difference in UVB and UVA1 p63 levels (p=0.001) (see Figure 4.3B). Mice expressing p63 exhibit a greater degree of re-epithelialisation and

expression of K5, K14 and loricrin (Parsa *et al.*, 1999) than p63 null mice. This chapter shows a UVR source specific difference only with K1 expression between UVA1 and UVB, in that UVA1 induces significantly more K1 than UVB at 3h. Involucrin showed peak expression with UVA1 and UVB at 24h but this was not significantly different from baseline. Perhaps we could have expected a statistically significant profile pattern of K1, K10 and involucrin expression in conjunction with p63 expression pattern in the basal epidermis with UVA1 and UVB.

Overall epidermal thickness was greater after UVB, which could represent a greater responsiveness of the skin to UVB rather than to biologically equal doses of UVA1. To support this we saw increased Ki67 expression at 3h by UVB but not UVA1 which could contribute to increased thickness.

Previous work however has shown that following 25mJ/cm^2 UVB there is increased K5 and K14 (as well as K6 and K19) gene expression *in vitro* however no changes in response to up to 20J/cm^2 of UVA (Bernerd *et al.*, 2001) have been reported, except an increase in K17 gene expression, mutations in which cause a skin and nail disorder (pachyonychia congenita (Eliason *et al.*, 2012)).

p63 associated p53 independent apoptosis

Both UVA1 and UVB induce CPD, at a given erythemal exposure (see Chapter 3). Cell death occurs by 3 mechanism; necrosis (which does not occur after UVR) (Godar, 1996; Godar *et al.*, 1994), immediate and delayed apoptosis (Godar *et al.*, 1994) which can occur following UVR. Immediate and delayed apoptosis by UVA1 are mechanistically different to that by UVB (Godar, 1999; Morita and Krutmann, 2000). Immediate apoptosis (<4h) (pre-programmed cell death) is UVA1 driven via

$^1O_2$ damage (Morita *et al.*, 1997) and also superoxide anions (Godar, 1999) likely via AP1, and downregulation of Bcl2 (Wang *et al.*, 1998). There is increased expression of Fas Ligand which initiates apoptosis by cross linking the Fas (Beattie *et al.*, 2005b). This is p53 independent (Breuckmann *et al.*, 2001).

UVR induced DNA damage is repaired through a p53 dependent pathway (Eller *et al.*, 1997; Smith and Fornace, 1997) and cells which cannot be repaired will undergo apoptosis (Sheehan and Young, 2002), which occurs at doses as low as 0.5MED UVB (Hofmann-Wellenhof *et al.*, 2004). Thus the skin also has a p53 dependent 'guardian-of-the-tissue' response to DNA damage and UVB stimulates induction of p53 (Breuckmann *et al.*, 2003; Godar *et al.*, 1994). p53 driven apoptosis produces SBC. Apoptosis then occurs by a common pathway involving FAS/FASL (CD95, APO1) and caspases (Yamauchi *et al.*, 2004).

UVA1 induces CPD *in vivo*, which needs a p53 dependent pathway for repair. At 1MED, our data showed hardly any p53 expression in the epidermis in response to UVA1, however at 3MED (*data not shown*) there was some expression seen by immunohistochemistry. UVA1 did not produce SBC at any dose. Previous work shows p53 formation after 2MED of UVA1 mainly in the basal epidermal (Burren *et al.*, 1998; Campbell *et al.*, 1993a), although other studies show a lack of UVA1 induced p53 or SBC (Beattie *et al.*, 2005b) at 3MED. Our work shows a lack of repair of CPD in the basal epidermis after UVA1. A possible explanation for this is that through an upregulation of p63 expression predominantly in the basal epidermis, p53 expression is suppressed resulting in the relative lack of downstream effects.

p63 mediated p53 suppression by UVA1

A hypothesis for our observations is shown in Figure 4.6. Our UVA1 dose (146J/cm^2), comparable to that used in high dose UVA1 phototherapy, increased p63 expression in the basal epidermis at 48h, which we feel is suppressing p53 expression particularly in this layer. This in turn suppresses apoptosis (seen as a lack of SBC) and G1/S arrest, also described by others after UVA (Runger *et al.*, 2012). Thus DNA damage is neither removed (by apoptosis) nor repaired. On the other hand, UVB (90mJ/cm^2) decreases p63 expression, which enables p53 protein expression, apoptosis, cell cycle arrest and CPD repair. Although there are mixed views on the carcinogenicity of UVA1 with some work suggesting that it is less carcinogenic due to a lack of p53 and SBC formation (Beattie *et al.*, 2005b), this chapter suggests that UVA1 may have more damaging effects than UVB *in vivo* as the persistence of CPD at the basal epidermal layer and a lack of p53 mediated apoptosis could mean that these CPD could be more likely incorporated as C→T mutations in further rounds of replication which over time could lead to skin cancers.

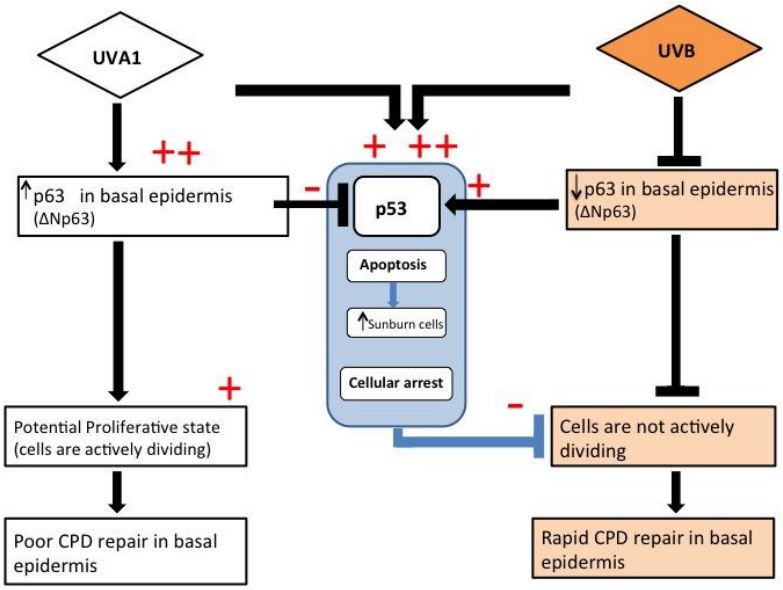


Figure 4.6 Summary of events (hypothesis)

Summary

The strong induction of p53 and apoptosis by UVB confirms p53's role as ''guardian of the genome'' (Ziegler *et al.*, 1993), but the weak induction of p53 and lack of apoptosis by UVA increases the probability of mutation by a CPD, especially given the lack of DNA repair in the basal layer. Although this study reports on TT widely regarded as non-mutagenic, UVA1 also induces C containing CPD with mutagenic potential. The data presented above suggest that these important differences maybe mediated by the differential response to p63 by the two spectra, which may also account for differences in the basal layer DNA repair responses.

These results may be significant in situations where UVA1 is used to achieve a biological endpoint such as tanning or a clinical endpoint in phototherapy. They may also have implications in sunscreen photoprotection. DNA repair systems evolved under solar radiation where most damage is caused by UVB. All sunscreens contain UVB filters, based on the erythema action spectrum. The data presented above suggest that some UVB may be important to trigger basal layer NER and that "over protection" from UVB may have some long-term disadvantages.

Chapter 5 : Downstream molecular changes and their functional importance after UVA1 irradiation, with emphasis on matrix metalloproteinases

Published paper:

Tewari A, Grys K, Kollet J, Sarkany R, Young AR.

Upregulation of MMP12 and Its Activity by UVA1 in Human Skin: Potential Implications for Photoaging.

Journal of Investigative Dermatology 2014: Oct;134(10):2598-609.

Abstract

Despite the predominance of terrestrial UVA1, its molecular effects on human skin *in vivo* are surprisingly poorly understood. In this chapter, time-dependent whole-genome expression, along with mRNA and protein changes in the skin after 1MED of spectrally pure UVA1 (50 J cm^2) and 300 nm UVB (30 mJ/cm^2) were examined. At 6h, genes induced to the greatest extent were those involved in the upregulation of the immune response through IL17 signalling for both UVA1 (p=3.148e-7) and UVB (p-5.1e-10) with the key upregulated pathways being those involved in inflammation, apoptosis and response to hypoxia. After 24h, the genes induced to the greatest extent were those involved in extracellular matrix remodelling (ECM) with both UVA1 (P=5.5e−7) and UVB (P=2.9e−22). There are source specific variations within this: UVB induces MMP1, MMP3 and MMP10 mRNA at 24h to a much greater extent than UVA1. Our specific finding was the induction of MMP12 by UVA1 at 24h. We saw a dramatic upregulation in the MMP12 microarray transcript in all individuals compared to UVB, and these findings were reproduced using the quantitative mRNA PCR experiments. We chose to also assess for the presence of MMP12 protein in the skin in a further 3 skin type I/II individuals and found MMP12 in human epidermis 24h post 1MED UVA1. A small amount of MMP12 protein was also produced by UVB. To try and understand its function in the skin we used insitu zymography staining techniques and found that UVA1 but not UVB induced MMP12 broke down elastin in the skin. This meant that only UVA1 induced MMP12 had elastase activity. We hypothesize that UVA1 induced MMP12 probably mediates some of the photoageing effects in the skin, particularly by contributing to elastin degeneration. We feel this is a process that likely occurs in

the later stages of solar elastosis and our results provide a possible mechanism for the UVA mediated process known as ‘skin sagging’. We conclude that MMP12 is also a good marker of UVA1 exposure.

5.1 Introduction

The effects of UVA1 on gene expression *in vivo* are poorly understood and have been much less studied than UVB (Enk *et al.*, 2006; Enk *et al.*, 2004). This is mainly because UVB was previously regarded to be more biologically important, and Chapter 3 discussed how this is no longer the case. Experiments to determine gene expression after UVA have been performed on reconstructed skin (Marionnet *et al.*, 2011; Marionnet *et al.*, 2010) although studies show that gene changes after *in vitro* and *in vivo* exposure are dissimilar (Enk *et al.*, 2006). Previous gene expression studies *in vivo* are limited and have primarily focused on expression differences between UVA and UVB in the pigmentation pathway (Choi *et al.*, 2010).

The hallmarks of long-term exposure to solar UVR are photoageing (Yaar and Gilchrest, 2007) and photocarcinogenesis (Sage *et al.*, 1996). In terms of photoageing, previous studies with UVB rich sources showed induction of MMP1, MMP3, and MMP9 mRNA (Brenneisen *et al.*, 1996; Fisher *et al.*, 1996; Fisher *et al.*, 1997) in human skin *in vivo* (see introduction). UVA sources have also been reported to induce MMP1 expression in fibroblasts (Herrmann *et al.*, 1993; Scharffetter *et al.*, 1991), however even <1% UVB contamination of a predominantly UVA source can be responsible for most of a given effect such as DNA damage (Woollons *et al.*, 1999) and there are few data on the effects of spectrally pure UVA1 on MMP in human skin *in vivo* (Wang *et al.*, 2013).

The aim of this chapter is to understand the molecular processes that lead to long-term effects of UVA1 on the skin by studying whole genome expression changes after a low dose acute exposure of pure UVA1, in comparison to UVB, in skin type

I/II individuals. The most significantly enriched pathway was found to be inflammation through Th17 signalling at 6h and extracellular matrix (ECM) remodelling at 24h. Although the mRNA results are discussed in detail, our most striking findings occurred with the ECM genes, thus protein and enzyme activity was studied for MMP only. In summary we report the results of a series of experiments following a 1MED dose of UVA1 on whole skin and analysis of DNA, RNA and protein changes that we believe has identified a novel marker of UVA1 exposure and might also explain some of UVA1's contribution to photoageing.

5.2 Materials and methods

This is described in detail in Chapter 2.

In brief 3 studies were performed: *Time course study*: 5 volunteers and biopsies taken 6h and 24h after 1MED UVA1. *Dose response study:* a further 5 volunteers and biopsies taken 6h after ¼, ½ and 1MED of UVA1. *UVA1 and UVB comparison study*: the 5 volunteers in the dose response study also had biopsies taken at 6h and 24h after 1MED of UVB and also a biopsy taken 24h after 1MED UVA1.

5.3 Results

5.3.1 Volunteer demographics

The demographics of volunteers used to study the genetic effects of UVA1 on human skin *in vivo* are shown in Table 2.1

5.3.2 Genes upregulated by UVA1 and UVB in human volunteers

We excluded volunteer 001 from the time course study as he was found to have an underlying autoimmune disease (Crohn's Disease), which skewed our results as upregulation of inflammatory genes, were also found at baseline. Thus the pooled intensities (n = 9 for UVA1 and n = 5 for UVB) of upregulated genes compared to each individual's non-irradiated control ($p<0.05$, fold change ≥ 2) were analysed for enriched pathways using Genego Metacore v7 and biological group known as Gene Ontology (GO) groups using Database for Annotation, Visualization and Integrated Discovery (DAVID) v 6.7 (Dennis *et al.*, 2003).

The main enriched pathway with low dose (sub-erythemal) UVA1 (12.5J/cm^2, 25J/cm^2) was upregulation of the immune response through T_H17 signaling. This was also the case after 50J/cm^2 UVA1 (1MED), and comparing gene changes with 30mJ/cm^2 UVB (1MED) the main upregulated pathway at 6h was upregulation of the immune response through T_H17 signalling for UVA1 (p=1.16e-6) and UVB (p=2.1e-4). Within this, for UVA1, key pathways of upregulated genes include inflammation (p=3e-3), apoptosis (p=3.82e-15) and response to hypoxia (p=1.988e-11). At 24h, the main upregulated pathway was ECM remodelling for UVA1 (p=5.5e-7) and UVB (p=2.9e-22). These pathways are part of a bigger network and the schematic below (Figure 5.1) shows the series of molecular events they trigger.

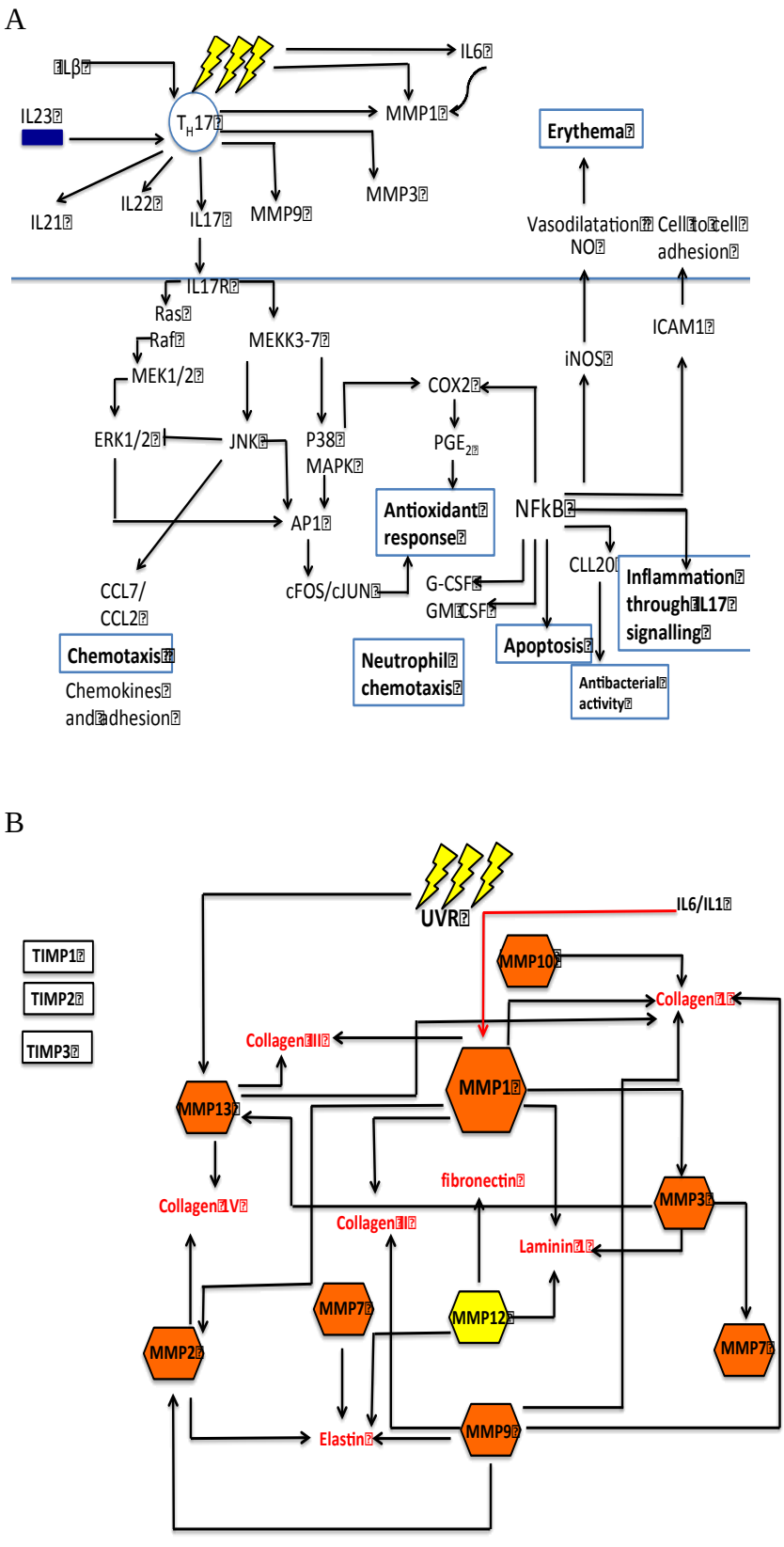
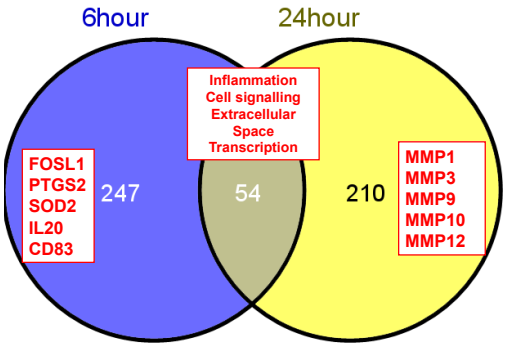


Figure 5.1 Top molecular signalling events 1MED post UVA1 and UVB
(A) Immune response through Th17 signalling at 6h showing an upregulation of key pathways (B) ECM remodelling at 24h demonstrating upregulation fo MMP1 and associated MMP.

The upregulated gene lists ($p \leq 0.05$, fold change ≥ 2) were also placed in Venn diagrams (Figures 5.2A-C) showing upregulated genes by UVA1, UVB or both at the two time points. Where relevant, these were clustered into Gene Ontology groups using DAVID (see Chapter 2 Materials and methods). 24h post UVA1, there was striking upregulation of MMP12. The individualised $\log_2$ transformed normalised intensity values for MMP12 are plotted in a heat map (Figure 5.3) which shows a highly upregulated MMP12 gene transcript by UVA1 at 6h and 24h.

A



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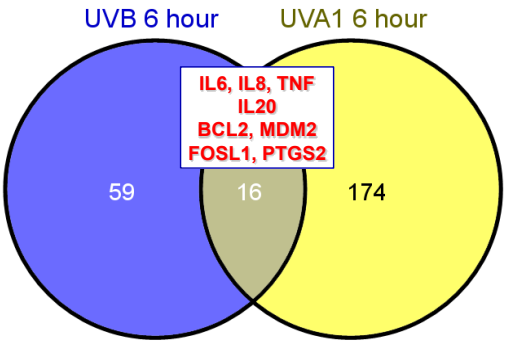

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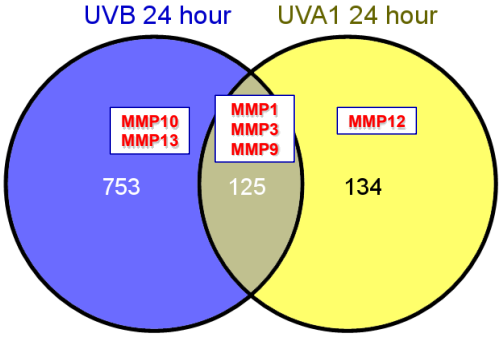


Figure 5.2 Venn diagrams demonstrating common and differentially upregulated genes (A) 6h and 24h post UVA1 (B) 6h post UVA1 and UVB (C) 24h post UVA1 and UVB

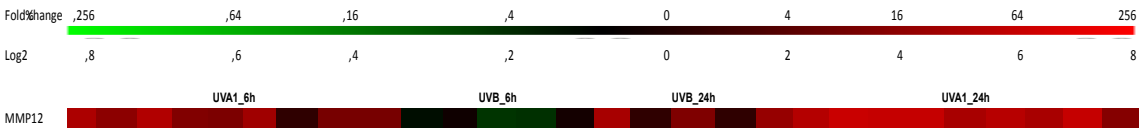


Figure 5.3 Heat map showing $\log_2$ fold change for MMP12
Expression values for each individual compared to unirradiated control sample (Red is up, green is down). Brighter intensity of red corresponds to a greater $\log_2$ fold change.

Figure 5.3A shows that different genes are upregulated by UVA1 at 6h and 24h. Figure 5.3B shows that UVA1 and UVB both upregulate apoptotic, inflammatory, antioxidant response and immunosuppressive gene clusters at 6h, whilst Figure 5.3C

presents a variation MMP upregulation by UVB and UVA1 at 24h. This data support the pathway bioinformatics analyses (Figure 5.1).

5.3.3 Down-regulated genes microarray analysis

Genes ≤ 2 fold and p≤0.05 were collected and plotted in the Venn diagrams below (Figure 5.4). Of the genes that were downregulated, most were at 24h by UVB (493 genes as opposed to 147 by UVA1) and UVB was particularly effective at down-regulating genes in epidermal development. Specifically these were found to be loricrin and filaggrin. UVB also downregulated MMP16. The rest of this chapter focuses on upregulated, genes and their functional importance.

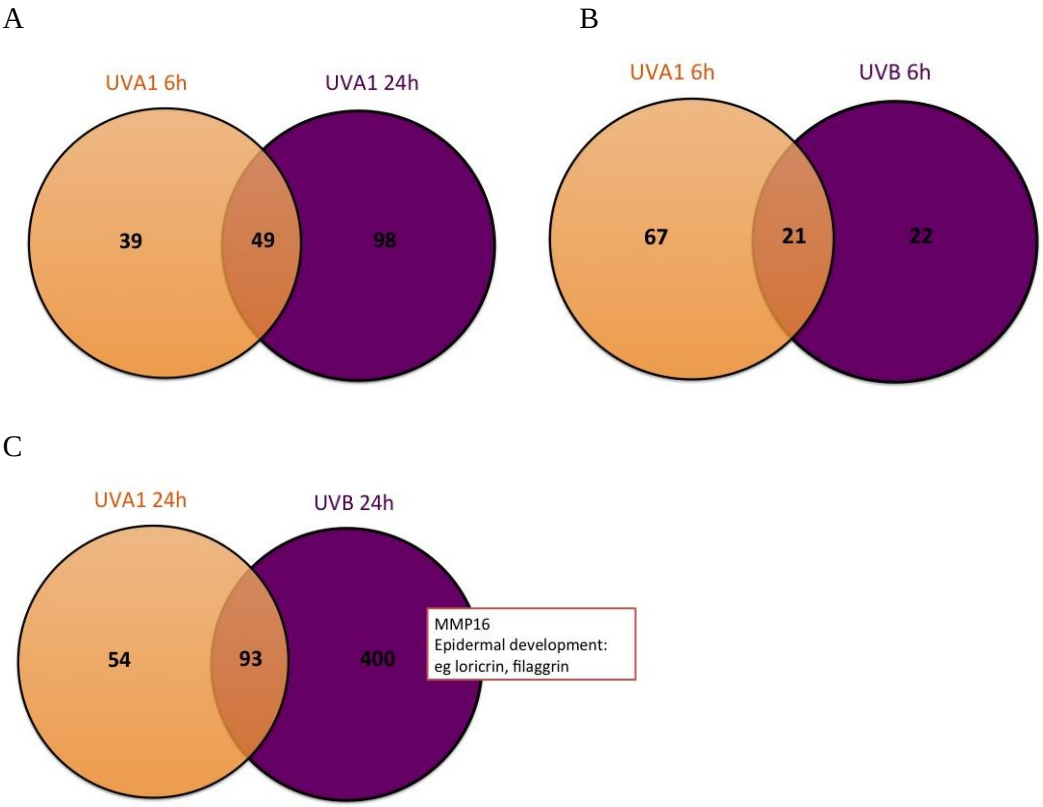


Figure 5.4 Venn diagrams demonstrating downregulated genes
(A) UVA1 at 6h and 24h (B) UVA1 and UVB at 6h (C) UVA1 and UVB at 24h.

5.3.4 ECM remodelling microarray analysis

Using the bioinformatics data, a microarray MMP gene expression analysis comparison at 6h and 24h was performed against unirradiated control samples. This enabled a numerical comparison of the data in Venn diagram (Figure 5.2C) and shows that transcripts encoding MMP1, MMP3, MMP9, MMP10, MMP12 are upregulated predominantly at 24h. MMP1, MMP3 and MMP10 are upregulated predominantly by UVB; 10 – 550 fold compared to 2-15 fold by UVA1 ($p \leq 0.05$, adjusted $p \leq 0.3$) at 24h. UVA1 induces MMP12 at 6h ~15fold, whilst UVB does not (~1, no fold change) and at 24h MMP12 is upregulated ~43 fold by UVA1 whilst UVB produces ~11 fold change ($p \leq 0.05$, adjusted $p \leq 0.3$).

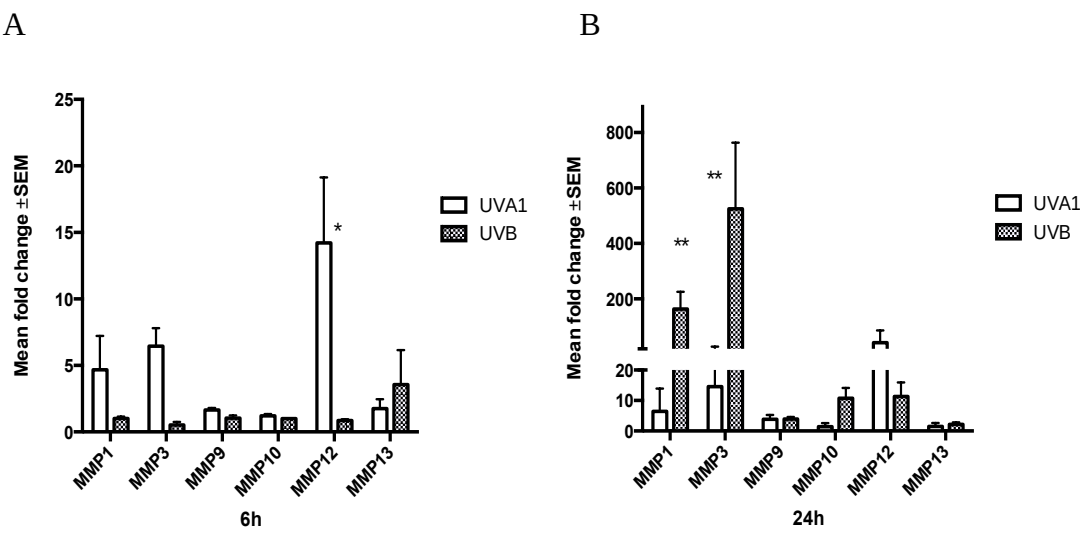


Figure 5.5 UVA1 and UVB gene expression changes at 6h (A) and 24h (B)

5.3.5 mRNA validation

Genes of interest that were validated by RTqPCR are shown below in Table 5.1 and were chosen according to generated upregulated pathway lists performed by GeneGo. For inflammation, antioxidant activity, apoptosis and ECM remodelling, an analysis of the microarray data using a large panel of individual genes (RTqPCR

array plates, Quiagen, Crawley, UK) produced a list of genes that were upregulated and could potentially be further examined by RTqPCR. The fold changes were placed in graphs and the top 4-5 genes in each group were chosen. No specific panel exists for immunosuppression, and as CD83 has been shown *in vitro* to positively upregulate IL10 expression (Kretschmer *et al.*, 2007) and was significantly upregulated in our data set, we decided to validate CD83 as well as IL10.

Gene bank	Gene Symbol	Gene Name/encoded protein
Inflammation		
M18403.1	IL6	Interleukin 6
Y00787.1	IL8	Interleukin 8
X02910.1	TNF	Tumour Necrosis Factor
AF224266.1	IL20	Interleukin 20
Immunosuppression		
M57627.1	IL10	Interleukin 10
NM_001251901	CD83	CD 83 molecule
Antioxidant activity		
NM_002133	HO1 (HMOX1)	Haem oxygenase 1
NM_005438	FOSL1	FOS like 1 antigen
NM_000963.1	PTGS2 (COX2)	Prostaglandin-endoperoxide synthase 2 (Prostaglandin G/H synthase and cyclooxygenase)
NM_006164	NRF2	Nuclear factor, erythroid 2-like 2
Apoptosis		
M59465.1	TNFAIP3	Tumour necrosis factor alpha-induced protein 3
M14745	BCL2	B-cell CLL/lymphoma 2
NM_006880	MDM2	MDM2 proto-oncogene, E3 ubiquitin protein ligase
AF307851	TP53	Tumour Protein 53
Extracellular matrix remodelling		
X54925	MMP1	Matrix metallopeptidase 1 (interstitial collagenase)
X05232	MMP3	Matrix metallopeptidase 3 (stromelysin 1)
NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
X07820	MMP10	Matrix metallopeptidase 10 (stromelysin 2)
L23808	MMP12	Matrix metallopeptidase 12 (macrophage elastase)
X75308	MMP13	Matrix metallopeptidase 13 (collagenase 3)

Table 5.1 Genes of interest for qPCR validation

5.3.6 Dose Response Study

Data below show that incremental doses of UVA1 produced increasing mRNA levels, linear regression p values in brackets.

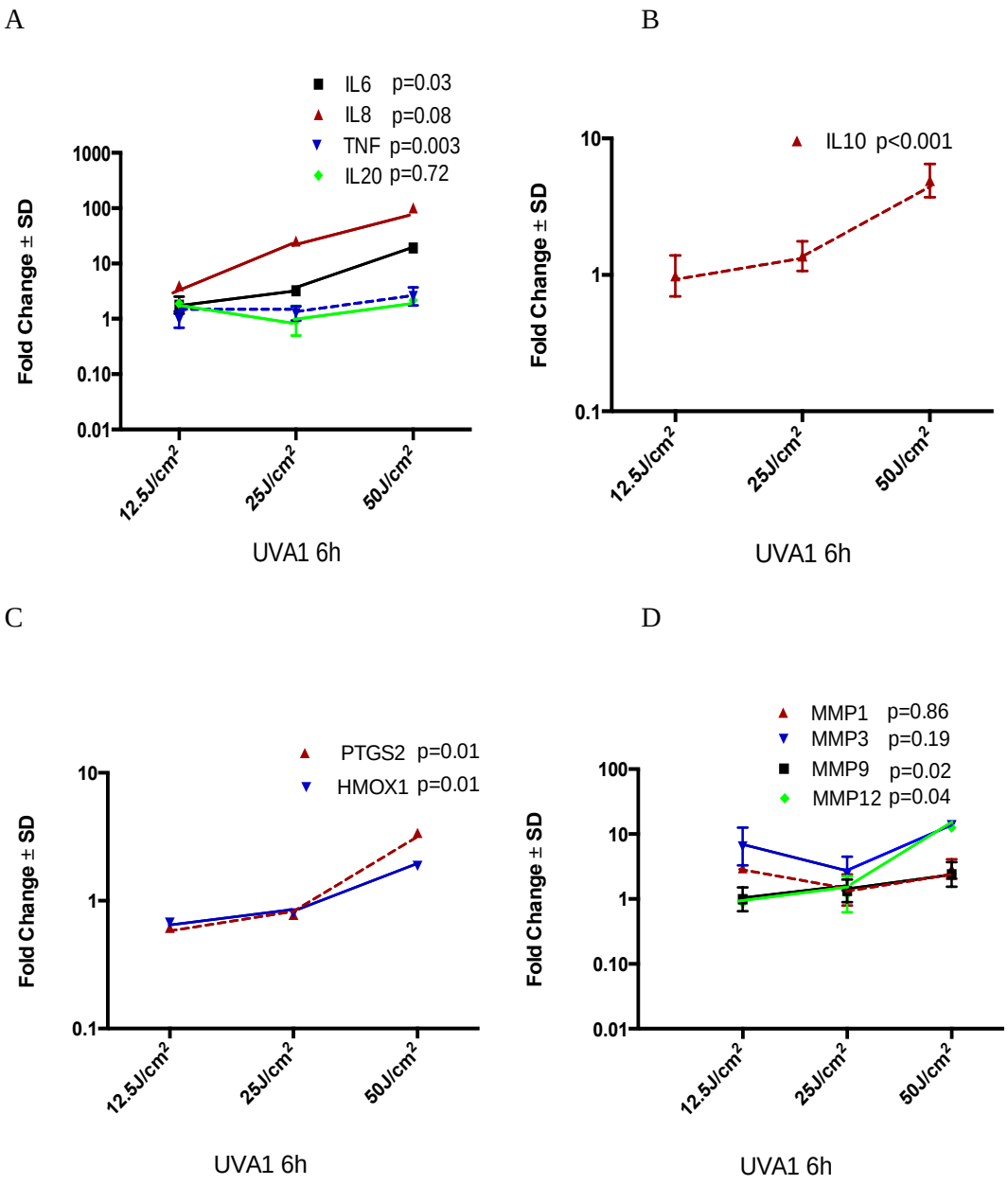
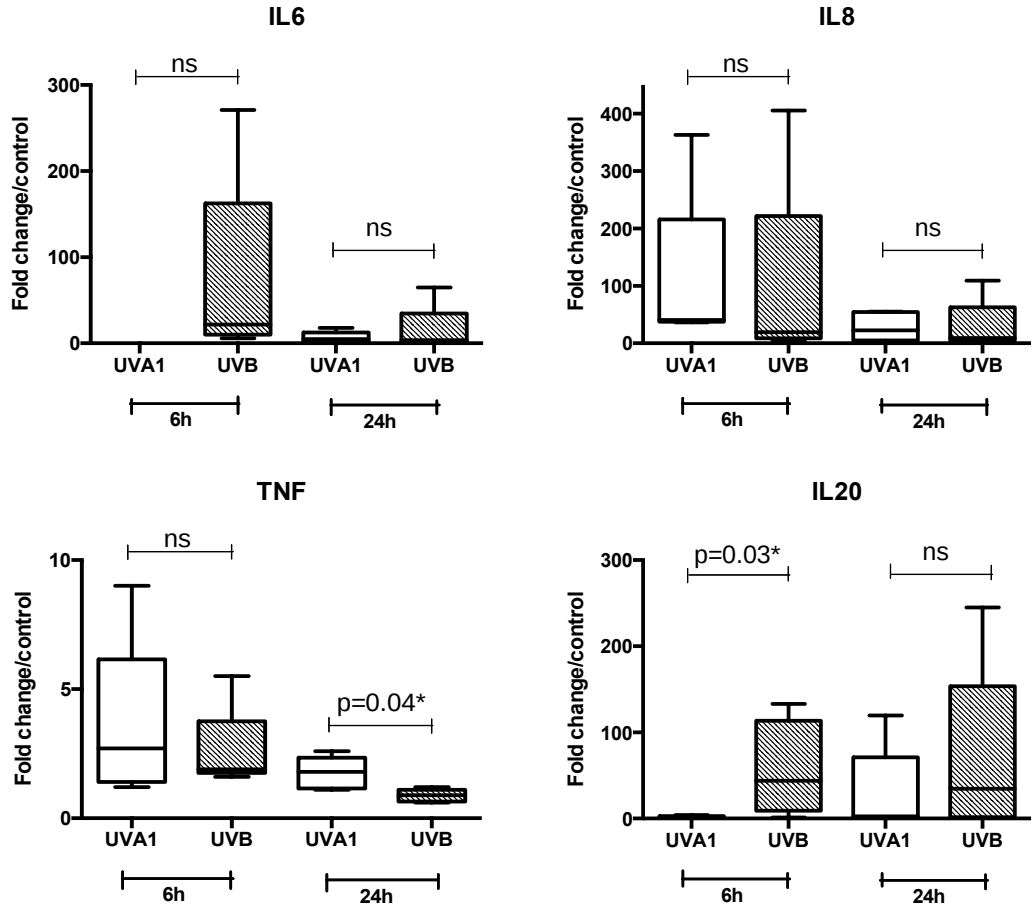


Figure 5.6 Dose response mRNA data
(A) inflammation (B) immunosuppression (C) antioxidant activity (D) ECM remodelling (MMP10 excluded)

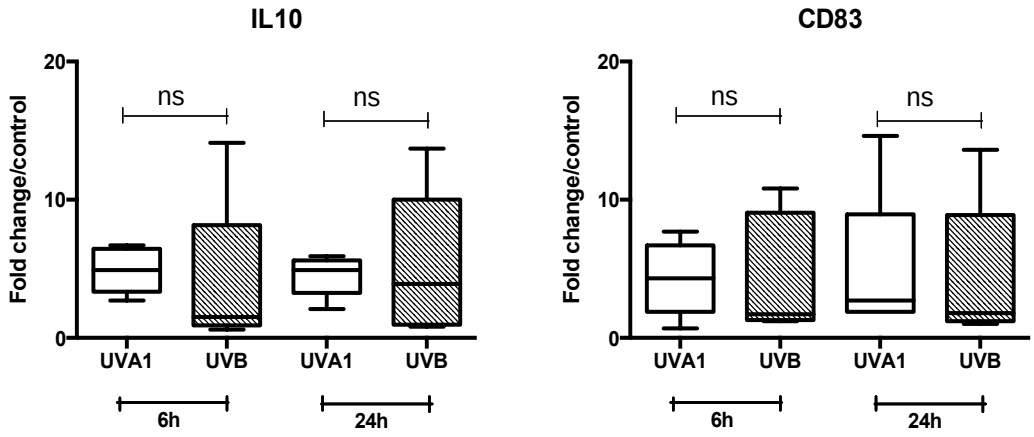
5.3.7 Time course study and UVA1 and UVB comparison study

Results for the time course study are placed together with the UVA1 and UVB comparision study (Figure 5.7). Results are presented as an individual fold change compared to each individual control (Table 5.2) as well as a mean fold change (Table 5.3).

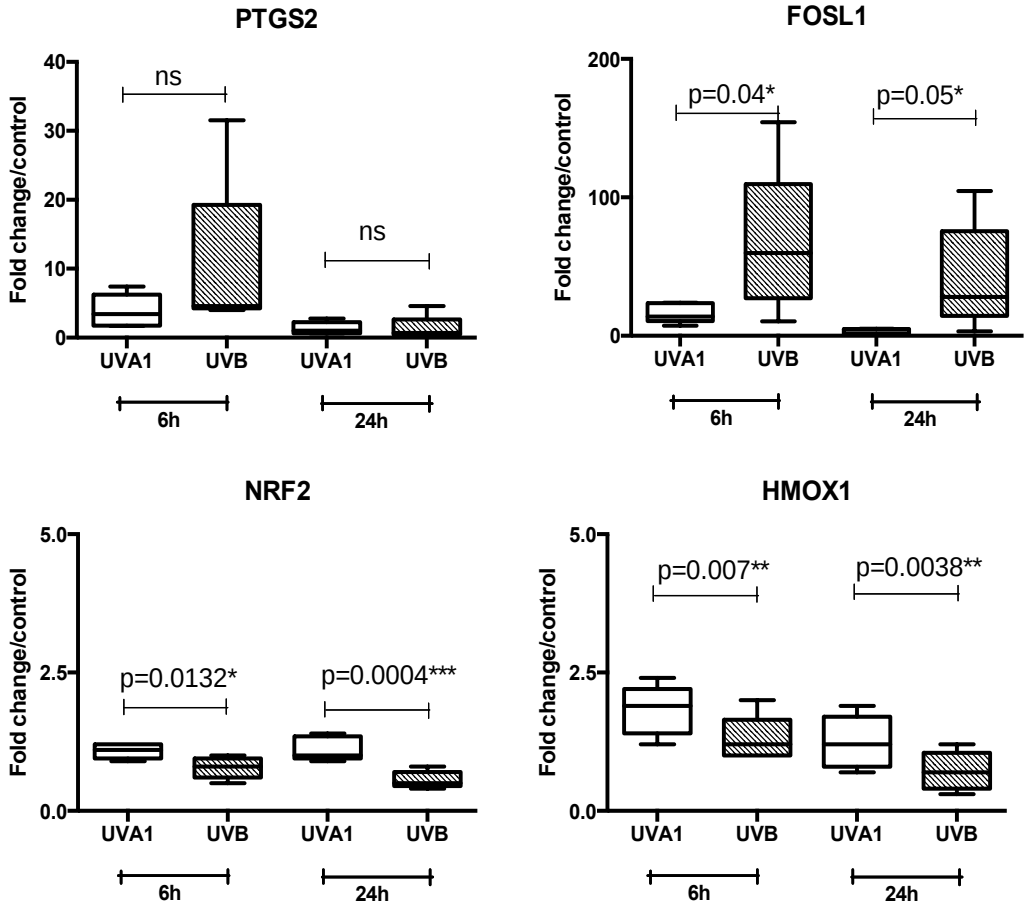
A Inflammation



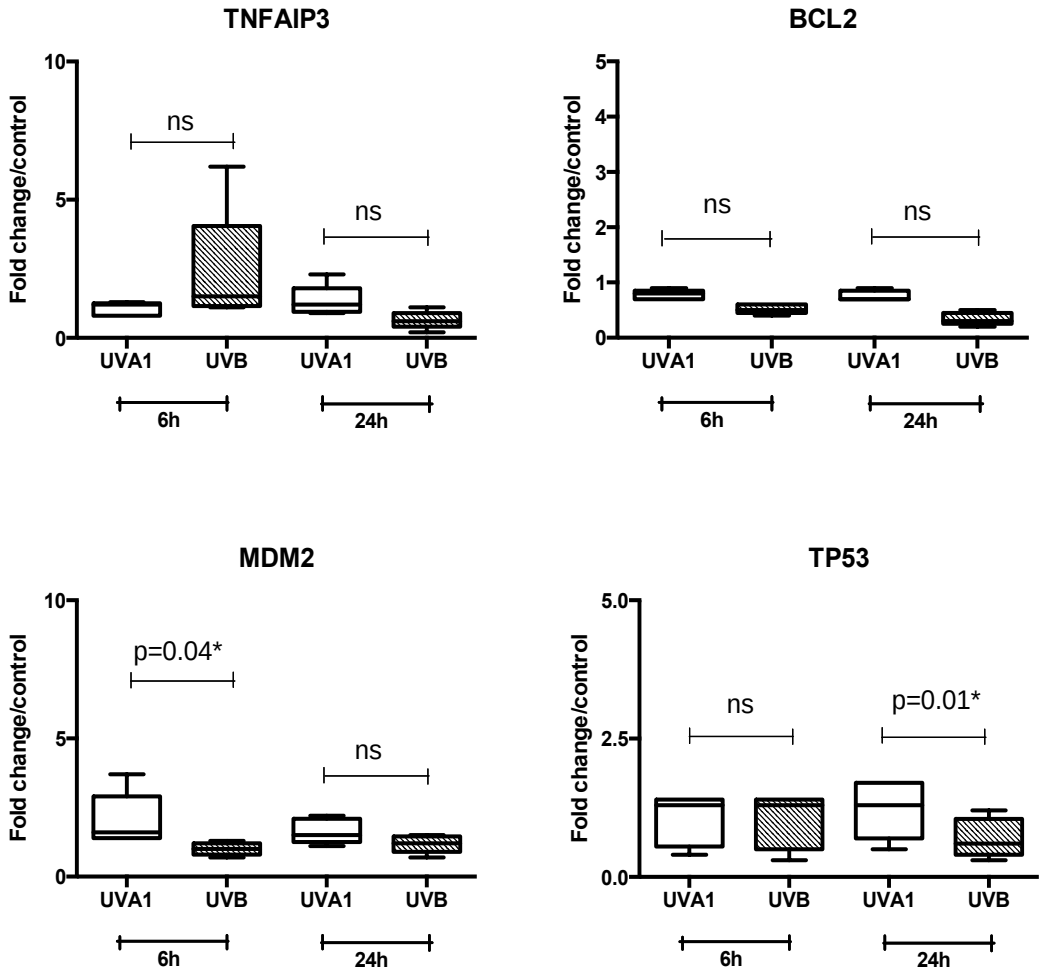
B Immunosuppression



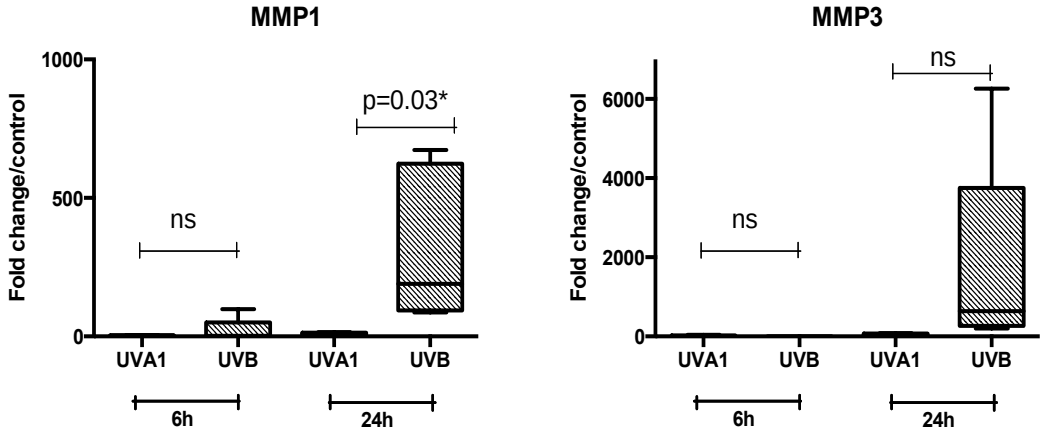
C Antioxidant activity



D Apoptosis



E ECM remodelling



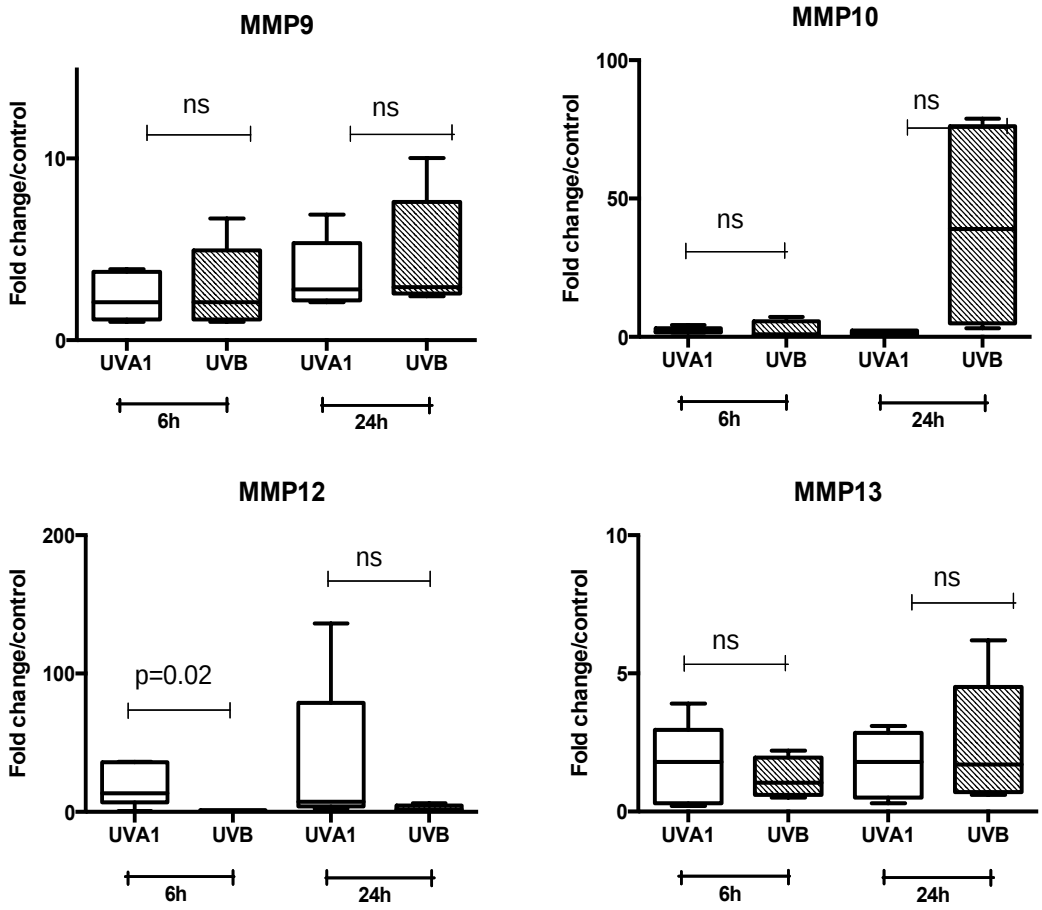


Figure 5.7 mRNA fold change at 6h and 24h post UVA1 and UVB
For genes involved in (A) inflammation (B) immunosuppression (C) antioxidant activity (D) apoptosis (E) ECM remodelling

Gene Name	Fold change (Treatment compared to unirradiated control)																			
Treatment	**UVA1 6h**					**UVB 6h**					**UVA1 24h**					**UVB 24h**				
Volunteer no	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Inflammation																				
IL6	**7.7**	**7.9**	**20.0**	**53.9**	**5.9**	**21.9**	**14.3**	**271.1**	**53.9**	**5.9**	1.3	**17.9**	**7.1**	**4.9**	1.2	**3.5**	**2.8**	**64.8**	**4.4**	1.2
IL8	**38.6**	**67.7**	**40.2**	**362.**	**36.3**	**19.4**	**13.3**	**405.4**	**38.1**	**4.15**	**8.5**	**22.7**	**54.0**	**55.3**	1.7	**16.0**	**4.2**	**109.4**	**9.2**	**5.8**
TNF	**2.7**	1.6	**3.3**	**9**	1.2	**2.0**	1.9	**5.5**	1.9	1.6	1.1	**2.1**	1.8	**2.6**	1.2	0.9	1.2	1.0	0.6	0.7
IL20	0.4	1.5	0.3	**4.0**	1.9	**32.4**	**55.1**	**133.2**	0.2	1.3	0.5	**119.5**	**22.9**	0.1	**2.5**	**34.5**	**62.0**	**245.0**	0.1	**2.9**
Immunosuppression																				
CD83	**5.7**	**3.1**	**7.7**	**4.3**	0.7	**10.8**	1.4	**7.3**	1.7	1.2	**14.6**	**2.7**	**3.3**	1.9	1.9	**13.6**	1.4	**4.2**	1.0	1.8
IL10	**6.7**	**4.0**	**2.7**	**4.9**	**6.2**	**2.2**	0.6	**14.1**	1.5	1.2	**4.9**	**5.9**	**5.3**	**2.1**	**4.4**	**6.3**	1.1	**13.7**	0.8	**3.9**
Antioxidant activity																				
HO-1	1.9	1.6	**2.4**	**2.0**	1.2	1.3	1.0	**2.0**	1.2	1.0	1.2	1.5	1.9	0.9	0.7	0.7	0.9	1.2	0.3	0.5
FOSL1	**23.9**	**7.3**	**23.4**	**13.9**	**13.9**	**64.8**	**59.8**	**154.2**	**43.6**	**10.4**	**4.7**	**5.1**	0.5	**4.5**	**2.8**	**46.4**	**28.1**	**104.6**	**3.2**	**25.5**
PTGS2	**3.4**	1.8	**7.4**	**5.1**	1.7	**7.0**	**4.0**	**31.5**	**4.6**	**4.5**	0.7	1.0	**2.8**	0.6	1.7	0.7	0.7	**4.6**	0.4	0.8
NRF2	1.0	1.2	1.2	1.1	0.9	0.5	0.9	0.8	1.0	0.7	1.0	1.4	1.3	1.0	0.9	0.5	0.8	0.6	0.4	0.5
Apoptosis																				
TNFAIP3	0.8	1.2	1.2	1.3	0.8	1.1	1.5	**6.2**	1.9	1.2	0.9	2.3	1.3	1.0	1.2	0.6	0.7	1.1	0.2	0.6
BCL2	0.8	0.9	0.7	0.7	0.8	0.5	0.5	0.6	0.4	0.6	0.7	0.9	0.7	0.7	0.8	0.3	0.4	0.3	0.2	0.5
MDM2	1.4	1.6	**3.7**	**2.1**	1.4	0.7	1.1	1.0	1.3	0.9	1.5	1.1	**2.0**	**2.2**	1.4	1.5	1.1	1.4	0.7	1.2
TP53	0.4	1.3	1.4	1.4	0.7	0.3	1.3	1.4	1.4	0.7	0.5	1.7	1.7	1.3	0.9	0.3	1.2	0.9	0.5	0.6
Extracellular Matrix																				
MMP1	**2.8**	**3.0**	**2.1**	**4.8**	1.8	**2.2**	1.5	1.9	0.7	**98.6**	**9.9**	**11.0**	**14.9**	**5.7**	**2.0**	**574.1**	**189.4**	**673.3**	**86.2**	**101.1**
MMP3	**16.7**	**5.0**	**3.2**	**40.3**	**3.1**	**4.8**	1.0	1.1	1.4	**2.1**	**36.0**	**10.8**	**65.5**	**82.9**	0.7	**1244.5**	**200.1**	**6263.0**	**639.4**	**327.1**
MMP9	**3.6**	1.3	1.0	**3.9**	**2.1**	**2.1**	1.0	**6.7**	**3.2**	1.3	**6.9**	**2.8**	**2.1**	**3.8**	**2.3**	**10.0**	**2.7**	**5.2**	**2.4**	**2.9**
MMP10	**4.3**	**2.0**	1.5	1.9	-	**7.2**	0.8	1.0	0.8	-	**2.3**	**2.1**	0.8	0.7	-	**67.8**	**10.1**	**78.9**	**3.1**	-
MMP12	**13.1**	**35.6**	3.9	**13.4**	**36.2**	0.5	1.2	0.2	1.2	0.8	**5.6**	**136.2**	**2.2**	**7.3**	**21.2**	**19.4**	**4.4**	**3.5**	0.8	**4.1**
MMP13	0.4	2.0	0.6	0.2	1.8	0.9	1.2	2.2	0.5	-	0.3	1.8	**2.6**	0.7	**3.1**	1.7	**2.8**	**6.2**	0.8	0.6

Table 5.2 Expression levels of genes assessed by RTPCR as a fold change compared to the unirradiated control sample

Inflammation	UVA1 6h	UVB 6h	UVA1 24h	UVB 24h
IL6	19.1	73.4	6.5	15.3
IL8	109.1	96.1	28.4	28.9
TNF	2.6	2.6	1.8	0.9
IL20	0.8	44.4	29.1	68.9
Immunosuppression				
CD83	4.3	4.5	4.9	4.4
IL10	4.9	3.9	4.5	5.2
Antioxidant activity				
HO-1	1.8	1.3	1.2	0.7
FOSL1	16.5	66.6	3.5	41.6
PTGS2	3.9	10.3	1.4	1.4
NRF2	1.1	0.8	1.1	0.6
Apoptosis				
TNFAIP3	1.1	2.4	1.3	0.6
BCL2	0.8	0.5	0.8	0.3
MDM2	2.0	1.0	1.6	1.2
TP53	1.0	1.0	1.2	0.7
Extracellular Matrix				
MMP1	2.9	21.0	8.7	324.8
MMP3	13.7	2.1	39.2	1734.8
MMP9	2.4	2.9	3.6	4.6
MMP10	2.4	2.5	1.5	40.0
MMP12	20.4	0.8	34.5	6.4
MMP13	1.0	1.2	1.7	2.4

Table 5.3 Summary of mean fold changes for all mRNA changes assessed at 6h and 24h

Key gene expression changes at 6h

Figure 5.7A shows that genes involved in inflammation (IL6, IL8, TNF) are equally upregulated by both UVA1 and UVB at 6h. Erythema is the clinical presentation of inflammation, thus with equivalent erythemal doses of UVB and UVA1 we might expect similar changes in inflammatory markers. It might be useful repeating experiments or increasing volunteer numbers to assess robustness of this finding.

The IL20 gene transcript was upregulated at 6h (~45 fold compared to baseline unirradiated by both spectra, *data not shown*). IL20 mRNA was upregulated by UVB at 6h (~50 fold, p=0.03) compared to UVA1, and both sources upregulated IL20 at 24h. IL20 is part of the IL10 subfamily and is upregulated in chronic inflammatory conditions such as psoriasis and rheumatoid arthritis (Ouyang *et al.*, 2011; Wegenka, 2010). Its upregulation following an erythemal exposure of UVB and UVA1 in this chapter probably occurs as part of the inflammatory response to UVR.

One of the key mediators of UVR induced immunosuppression is IL10, produced from T_H2 cells and also released from T_{reg} cells. Both UVA1 and UVB appear to induce similar immunomodulatory effects as CD83 and IL10 were upregulated to similar levels at 6h and 24h (~4-5 fold above baseline unirradiated levels). If erythemally equivalent doses of UVA1 and UVB result in comparable upregulation of IL10, it might suggest that the erythemal response is a trigger for IL10 induction and consequent immunosuppression. Other work however shows that 3MED UVB produced significant amounts of IL10 whilst UVA1 did not (Skov *et al.*, 1998). The impact of immunosuppression by UVB and UVA1 could be further studied by also examining IL4, and possibly IL12 levels which we would expect to be lower in immunosuppressed states (Krutmann *et al.*, 1992; Krutmann and Morita, 1999; Schwarz, 2005).

A heat map of upregulated AOx and ROS metabolites can be found in Appendix C where different AOx response genes are upregulated by both UVA1 and UVB at 6h and 24h. Following UVR exposure, a rapid cellular AOx response is induced in the skin after UVR exposure with activation of constituent GPX, SOD1, catalase as well

as Haem oxygenase (HO)1 and ferritin (Wondrak *et al.*, 2006). HO is an essential enzyme in haem catabolism and occurs as an inducible HO1 form and a constitutive HO2 form (Tyrrell, 2004). Studies in cultured human skin fibroblasts show the induction of a specific stress protein by UVA (at 334-365nm) and near visible radiation (405nm) that was minimally induced at shorter UVB wavelengths (Tyrrell, 2004) and it is felt that HO1 plays a role in the UVA oxidative damage response (Kielbassa *et al.*, 1997; Mouret *et al.*, 2006). Absorption of UVA by haem containing chromophores (such as cytochromes and protoporphyrin IX) produces free radicals such as $^1O_2$ (Basu-Modak and Tyrrell, 1993; Tyrrell and Pidoux, 1989). In order to stabilise the haem ring, energy is transferred to a Nrf2/Keap1 complex which then results in HO1 activation. HO1 transcription can also occur in the dermis via haem binding to Bach1 repressor protein. In our data set, UVA1 appears to significantly upregulate both HO1 (6h p=0.007 and 24h p=0.0038) and NRF2 (6h p=0.0132 and 24h p=0.0004) compared to UVB although the fold changes were small (less than 2.5 fold).

Our data show that PTGS2 (or cyclooxygenase 2, COX2) is principally upregulated by UVB at 6h (see Figure 5.7C), however the large inter-person variability probably explains why there is no statistical significance. PTGS2 is upregulated by UVB (Isoherranen *et al.*, 1999) and UVA via p38 MAPK transcription (Bachelor *et al.*, 2002) and converts arachidonic acid to prostaglandin H2 (PGH_2), an important precursor of prostaglandins (PGD_2, PGE_2, PGF_{2a}), prostacyclin (PGI_2) and thromboxane A2. These play roles in vasodilation and inflammation (Brooke *et al.*, 2013; Rhodes *et al.*, 2009). PTGS2 is a mediator of UVR inflammation, as its inhibitors such as indomethacin suppress UVR induced erythema (Ibbotson *et al.*,

1996). Elevation of prostaglandin E_2 (PGE_2) levels are thought to play a role in carcinogenesis through inhibition of apoptosis, promotion of angiogenesis and increased cell proliferation (Fosslien, 2000). Arachidonic acid is increased in oxidative stress situations (Balboa and Balsinde, 2006) probably explaining how PTGS2 is classified as an antioxidant activity gene.

FOSL1 was strikingly upregulated by UVB compared to UVA1 at 6h (UVB/UVA1 fold difference 4, p=0.04) and 24h (UVB/UVA1 fold difference 11 p=0.05). It is upregulated in inflammatory conditions such as psoriasis and binds to c-JUN forming the transcription factor complex AP1 that regulates cell proliferation, differentiation and MMP transcription. AP1 transcription is ROS inducible. Our recent findings of FOSL1 expression with biologically significant doses of UVB and UVA are novel and suggest a significant contribution from UVB as well as UVA in induction of oxidative stress.

A preliminary mRNA assessment of 4 apoptosis genes (TNFAIP3, MDM2, BCL2 and TP53) did not show any UVR source specific differences at either 6h or 24h. There was minimal upregulation of TP53 mRNA given that in Chapter 4 we demonstrate UVB induced p53 protein expression in the epidermis. Work however suggests that p53 protein expression increases following post translational modification of the protein (Appella and Anderson, 2001) rather than directly via mRNA translation although mRNA levels might otherwise be expressed at different time points to what we examined.

UVA1 and UVB induced ECM remodelling

UVB upregulates more MMP1 mRNA (UVB/UVA1 fold difference 13.2 p=0.0062), MMP3 (UVB/UVA1 fold difference 38.7 p=0.0016), and MMP10 (UVB/UVA1 fold difference 27.1 p=0.028) at 24h compared to UVA1. UVA1 upregulates MMP12 mRNA at 6h (UVA1/UVB fold difference 19.2 p=0.02) and also at 24h (UVA1/UVB fold difference 8.26 p=0.22), although at 24h there is large inter-person variation and the difference is not significant. Both UVA1 and UVB induce MMP9 mRNA at 24h (~5fold compared to baseline) with no statistical difference in upregulation (6h p=0.33, 24h p=0.12) (Figure 5.7E).

5.3.7.1 Protein validation Study: Immunofluorescence and *in situ* zymography

In a further 3 volunteers (see methods) we chose to study MMP1 and MMP12 protein in the skin. MMP1 is the main ECM enzyme found in the dermis: studies show that a blocking antibody to MMP1 removed ~ 95% of collagenolytic activity in UVR-exposed human skin (Brennan *et al.*, 2003). Collagen I is the main substrate for MMP1 and collagen IV and elastin are substrates for MMP12. We also examined enzyme activity (collagen I, collagen IV and elastin substrate breakdown) using *in situ* zymography (ISZ).

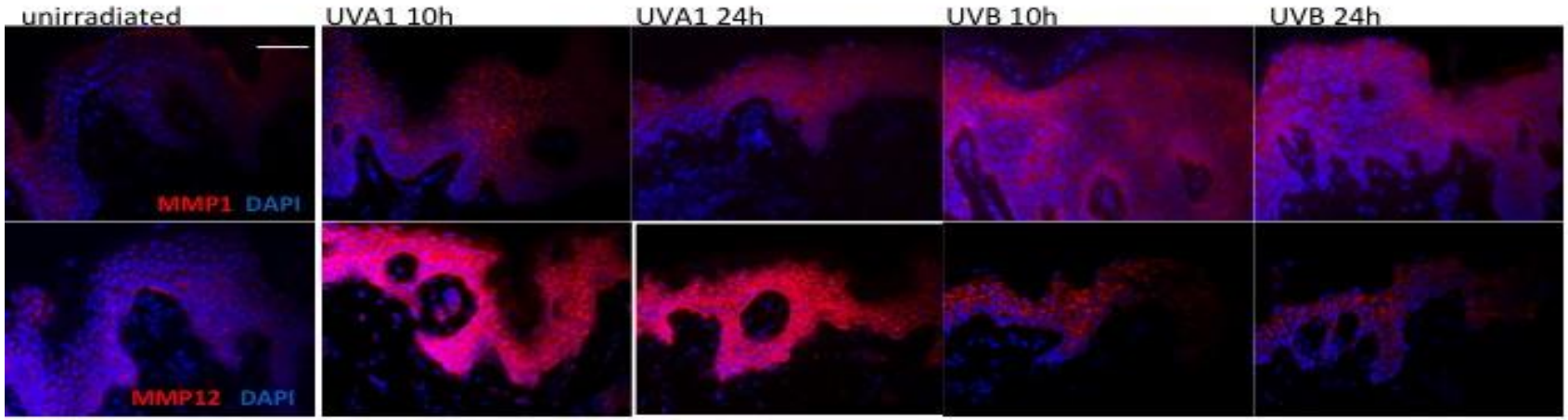
Examples of protein staining (MMP1 and MMP12) and ISZ (DQ™ collagen I and IV) are shown in Figures 5.8A and B. UVB and UVA1 induce similar levels of MMP1 protein at 10h and 24h (Figure 5.9A). DQ™ collagen type I hydrolysis as indicated by green fluorescence, occurred predominantly after 1MED UVB at 24h compared to 1MED UVA1 (p=0.031) (Figure 5.9B). As collagen I is also a substrate for MMP2, MMP3, MMP9, MMP13 (Yan and Blomme, 2003), we did

further immunostaining to assess for MMP2 and MMP9 protein (Figures 5.9C and D). MMP2 is not formed after UVA1 or UVB, and only UVB induces a significant upregulation in MMP9 at 24h as shown in previous studies (Fisher *et al.*, 1997). UVB and UVA1 induce similar levels of MMP13 mRNA (*data not shown*), thus the spectral difference that we detected for DQ™ collagen type I hydrolysis is indicative of MMP1 and also probably some UVB mediated MMP3 and MMP9.

UVA1 preferentially induces MMP12

MMP12 protein is formed by UVA1 at 24h ($p=0.04$). A lesser degree of induction of MMP12 protein is seen with UVB at 10h and 24h, despite the absence of MMP12 mRNA induction at 6 and 24h. UVA1 was more effective than UVB for the degradation of DQ collagen type IV in the epidermis (10h, $p=0.027$). This is primarily a substrate for MMP12, but it is also hydrolyzed by MMP2, MMP7, MMP9, and MMP25 (Chakraborti *et al.*, 2003; Yan and Blomme, 2003). Our data showed that these other MMP were not induced by UVA1, thus the induction of enzyme activity is most likely to reflect MMP12 activity. There is some collagen IV hydrolysis with UVB at 10h and 24h that could be due to UVB-induced MMP9 (Figure 5.9F), although without MMP12-blocking enzymes we cannot rule out the effects of MMP12. Figure 5.9G shows that UVA1 induces elastin breakdown that is not seen with UVB. MMP12 is the most important enzyme for the degradation of elastin (Gronski *et al.*, 1997; Woessner, 1991) although it shares its elastolytic activity with MMP2, MMP7 and MMP9, and breaks down other substrates including type IV collagen (as shown here), laminin 1, fibronectin, vitronectin, and proteoglycans (Gronski *et al.*, 1997).

A



B

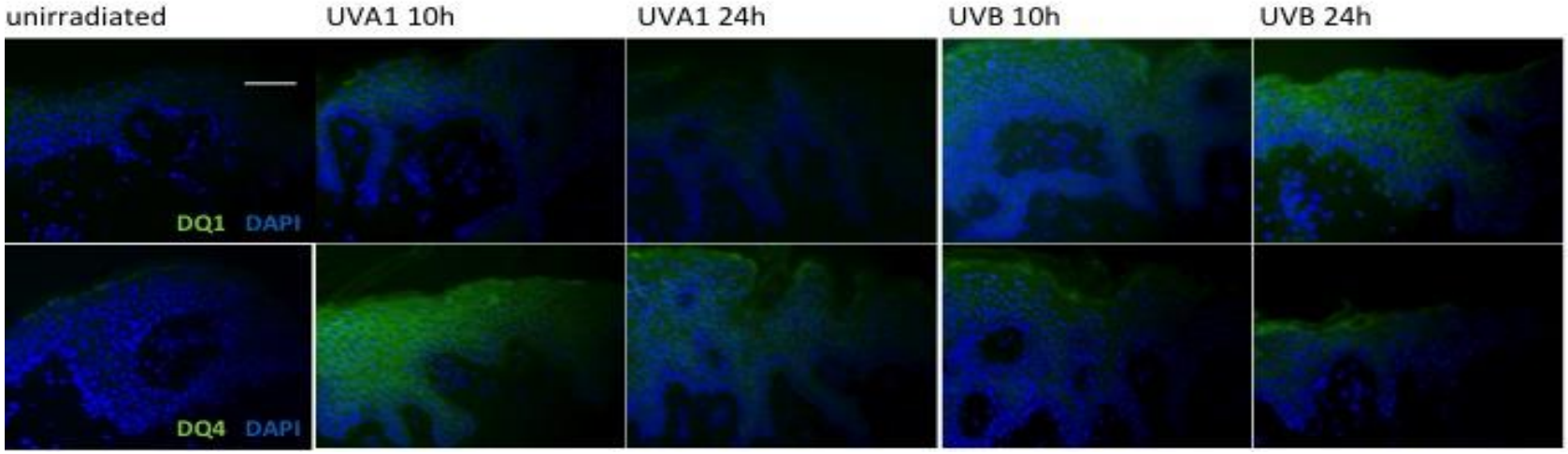
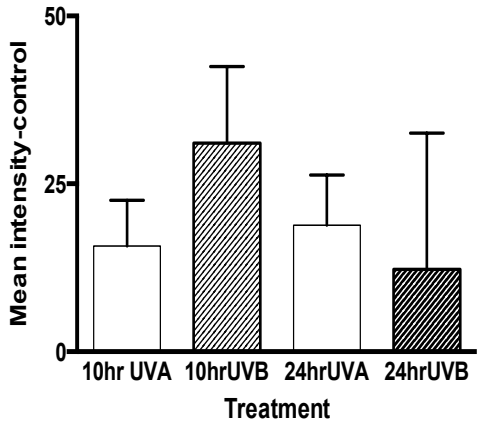
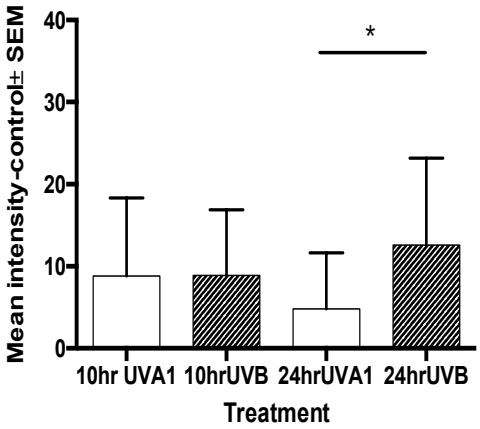


Figure 5.8 Examples of (A) MMP1, MMP12 and (B) DQ1 and DQ4 staining

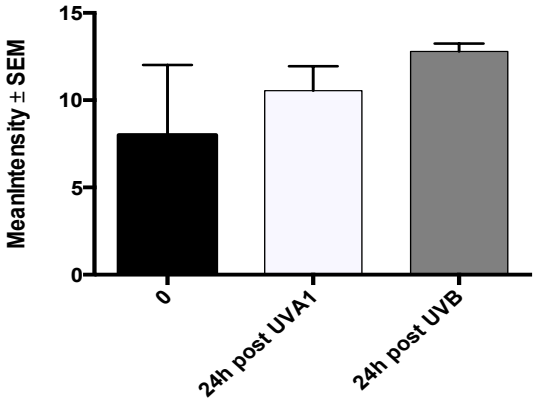
A MMP1



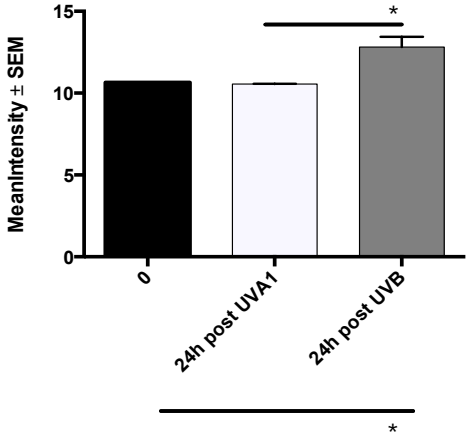
B DQ1



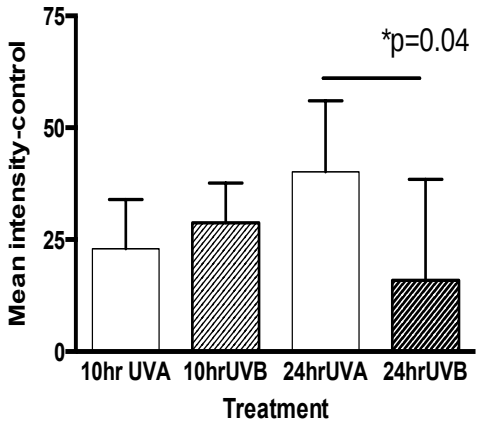
C MMP2



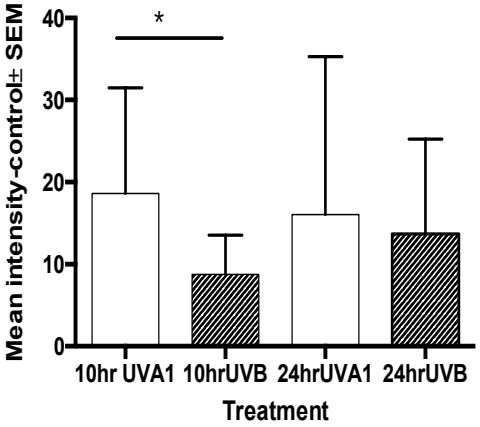
D MMP9



E MMP12



F DQ4



G Elastin

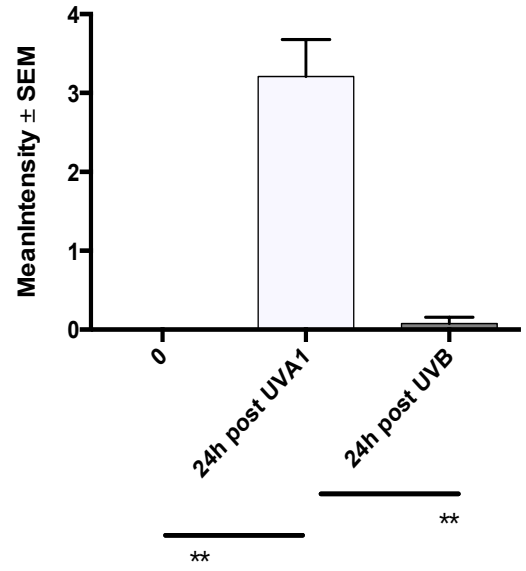


Figure 5.9 Epidermal protein and enzyme activity quantification
(A) MMP1 (B) DQ1 breakdown (C) MMP2 (D) MMP9 (E) MMP12 (F) DQ4 breakdown (G) Elastin breakdown

Differential expression of MMP1 and MMP12 proteins within the epidermis

The intensity of MMP1 and MMP12 was examined across the epidermis in order to given an insight into mechanism of induction. Figure 5.10A and B show that MMP1 protein is differentially expressed within the epidermis (upper vs. lower epidermis UVB p=0.005 and UVA1 p=0.01) with more produced in the upper layers of the skin, but there is no specific pattern of MMP12 expression across the epidermis (Figure 5.10C and D) (upper vs. lower epidermis UVB p= 0.20, UVA1 p=0.42).

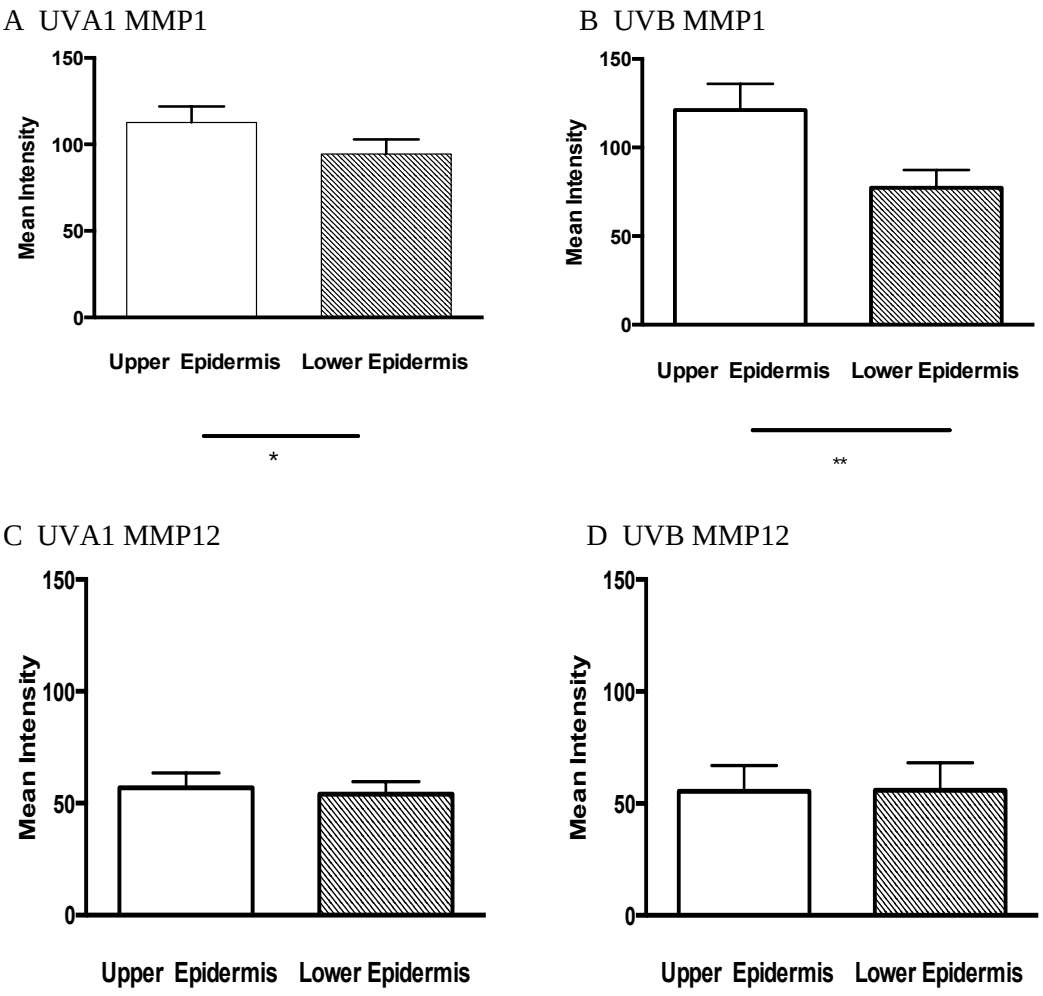
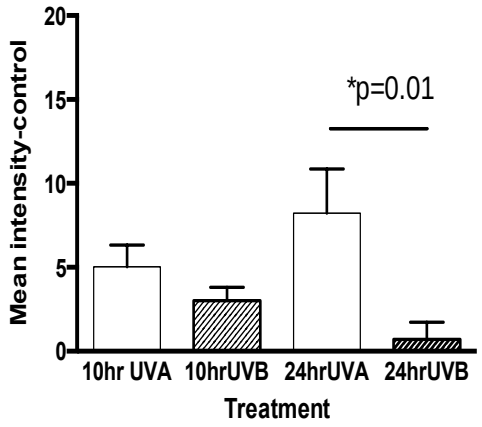


Figure 5.10 Differential expression of MMP1 and MMP12 across the epidermis

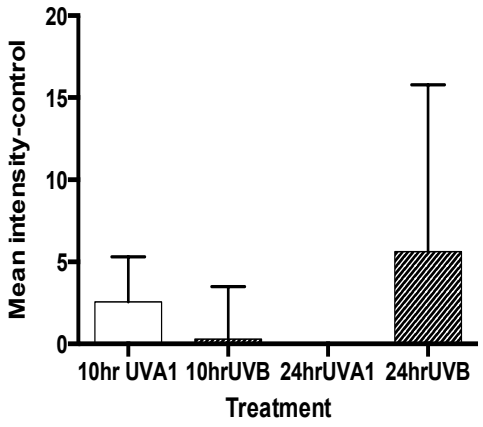
UVR induced MMP and its activity in the dermis

MMP1 and MMP12 protein formation in the dermis are shown in Figures 5.11A-D. Protein expression is significantly greater for UVA1 than UVB for MMP1 and MMP12 (A and C) but there are no spectral differences for enzyme activity (B and D). Overall, there is a greater abundance of protein and activity for both MMP1 and MMP12 in the epidermis.

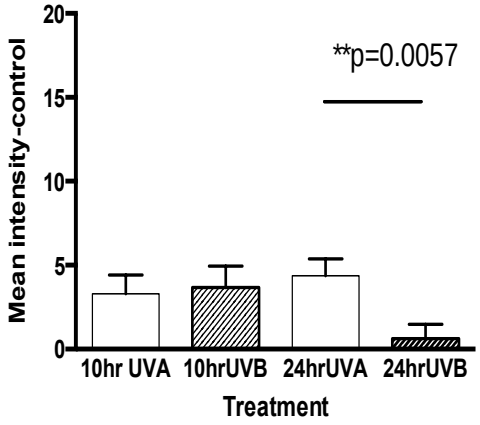
A MMP1 Dermis



B DQ1 Dermis



C MMP12 Dermis



D DQ4 Dermis

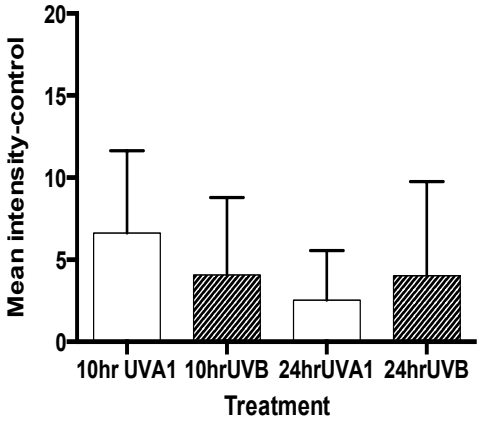


Figure 5.11 Dermal MMP1, MMP12 protein and activity

Macrophages in the skin are a potential source of MMP12

Figure 5.12 shows significant depletion of dermal macrophages (CD68+) 24h after UVB exposure (p=0.01). In contrast, UVA1 had no effect on dermal macrophage numbers (p=0.21) compared with non-irradiated controls.

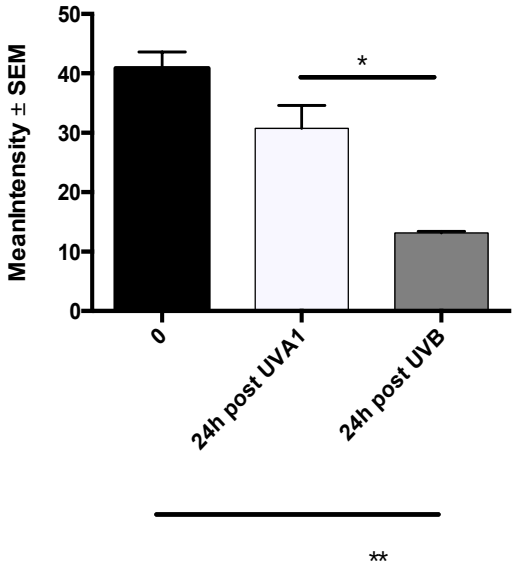


Figure 5.12 CD68 staining at 24h post UVA1 and UVB

5.4 Discussion

Our gene array data, supported by RTqPCR, show that an erythemal exposure of UVB and UVA1 readily induce a range of processes at 6h, and MMP predominantly at 24h.

Our RNA studies show that 1MED UVB in skin types I/II individuals induces more MMP1 and MMP3 mRNA (350 and 1700 fold) in the epidermis compared to UVA1 (25 and 40 fold) (Figure 5.7), and our UVA1 data are comparable (given the error range in both data sets) to other work where UVA1 increased MMP1 and MMP3 mRNA by 80- and 50- fold, respectively at 24h (Wang *et al.*, 2013). Our recent study looking at MMP1 mRNA expression at erythemal doses of SSR, UVB and UVA showed no difference between UVA and UVB induced MMP1 mRNA (Tewari *et al.*, 2012), although the UVA source (UVASUN) emits small amounts of UVA2 that may have accounted for the results.

We found no difference in MMP1 protein levels after UVA1 and UVB, and MMP1 was predominantly formed in the epidermis, which is expected because of its role in tissue homeostasis after UVR injury. We also show that at 24h, MMP1 and MMP12 proteins are more readily induced in the dermis by UVA1 than UVB which is supported by other work demonstrating the sensitivity of fibroblasts to UVA *in vitro* (Scharffetter-Kochanek *et al.*, 1993) and *in vivo* (Scharffetter *et al.*, 1991) but is also probably due to differential spectral penetration into the skin *in vivo* (Tewari *et al.*, 2012; Tewari *et al.*, 2011b). This may also explain the effectiveness of UVA1 phototherapy in collagenolysis for diseases such as scleroderma, sclerodermatous GVHD, nephrogenic systemic fibrosis, systemic sclerosis and lichen sclerosis (Kerr *et al.*, 2012b). Importantly, we show that UVA1, but not UVB, induces elastin degradation at 24h (Figure 5.9G), which can be attributed to the induction of MMP12. Interestingly the small amounts of MMP12 produced by UVB did not appear to cause elastin breakdown which raises the question of whether UVB induced MMP12 was either a non specific result or it is produced in insufficient amounts to cause elastin breakdown. This, in association with our data, strongly suggests that MMP12 is a UVA1 specific biomarker and responsible for elastin breakdown in the skin.

MMP12 producing cells

MMP12 (macrophage metalloelastase (MME), human macrophage elastase (HME)) (Kahari and Saarialho-Kere, 1997) is one of 8 elastases that contribute to elastic recoil in skin, blood and lung tissue (Liang *et al.*, 2006). It is produced predominately by macrophages in response to tissue injury (Shapiro, 1998; Shapiro *et al.*, 1993) and is also expressed by T cells (Hughes *et al.*, 1998), transformed

keratinocytes and keratinocyte derived tumours (Kerkela *et al.*, 2000). We found more macrophages in the dermis after UVA1, as UVB induced significant macrophage depletion at 24h ($p=0.02$) (Figure 5.12), which could contribute to the higher levels of UVA1-induced MMP12. However, its predominant epidermal location in our data suggests that after erythemal doses of UVA1 and UVB, keratinocytes produce the majority of MMP12 in the skin. MMP12 stimulates TNFα production (Chandler *et al.*, 1996), thus its predominance in the epidermis might be important in inflammation. It is also upregulated in psoriatic tissue (Suomela *et al.*, 2001), an often described TNF mediated disease. Other work has shown an upregulation of MMP12 mRNA in the epidermis and dermis in response to UVR (Chung *et al.*, 2002) with a mixed UVA/UVB source.

MMP12 is the major elastinolytic MMP in the skin (Gronski *et al.*, 1997; Senior *et al.*, 1991) although other elastinolytic MMP in the skin include MMP2, MMP7 and MMP9 (which were not upregulated in our data set after UVA1). It is a known major contributor to remodelling of elastotic tissue in sun-damaged skin (Saarialho-Kere *et al.*, 1999) as is generally found in areas devoid of normal elastic fibres (Vaalamo *et al.*, 1999). In contrast, MMP9 is found in regions of less damaged elastic fibres with morphologically better preserved collagen (Vaalamo *et al.*, 1998), whilst MMP2 is seen in more elastin rich areas (Vaalamo *et al.*, 1999) suggesting an association of MMP12 and elastin degeneration. Other work has also shown MMP12 accumulation in extensively photoaged dermis (Chung *et al.*, 2002).

Other elastases in the skin include neutrophil elastase (NE) and UVR induced NE causes ECM breakdown (Li *et al.*, 2013; Takeuchi *et al.*, 2010), but the relative contributions of UVA1 and UVB to its formation in the skin is not known. Interestingly NE deficient mice are resistant to photoageing (Starcher and Conrad, 1995; Takeuchi *et al.*, 2010) and SCC formation (Starcher *et al.*, 1996) (Wulf *et al.*, 1989) suggesting important pathogenic links between the two processes.

Depth of MMP in the skin

DNA is a chromophore for MMP1 via CPD formation (Dong *et al.*, 2008). Our recent findings (Tewari *et al.*, 2012; Tewari *et al.*, 2011b) showed attenuation of UVB induced CPD with skin (epidermis and dermis) depth due to UVB absorbing chromophores in the epidermis. The attenuation of MMP1 as shown in Figures 5.10A and B suggests a direct role of UVB in its formation, possibly also mediated via CPD. The distribution of MMP12 is different from MMP1, suggesting a different chromophore, with MMP12 probably being mediated by ROS formation (Scharffetter-Kochanek *et al.*, 1993; Wlaschek *et al.*, 1995). Studies show that the application of ROS scavengers reduced MMP12 mRNA in human skin (Chung *et al.*, 2002).

Location of MMP in the skin

In this Chapter we show that MMP protein localises in the epidermis although we know that dermal extracellular collagens are degraded by MMP in pathological processes such as photoageing. Other work shows that acute UVR exposures produce predominantly epidermal MMP (MMP1, MMP3, MMP9) (Quan *et al.*, 2013; Quan *et al.*, 2009), which is thought to diffuse into the dermis to degrade collagen. In chronically sun exposed skin such as in studies on photoaged skin,

more MMP is found in the dermis than in the epidermis (Chung *et al.*, 2002; Quan *et al.*, 2013) which overall suggests that repeated solar UVR exposure results in an accumulation of dermal MMP, whether by diffusion from the epidermis or by a gradual accumulation of dermally synthesized protein. It is possible that our 24h sampling time was not optimal for MMP12 diffusion from the epidermis into the dermis whose peak formation occurs 48h post exposure (Chen *et al.*, 2005).

The role of MMP12 in cancer and disease

MMP12 is produced by epithelial SCC and BCC, and is found more abundantly in less differentiated SCC, whilst premalignant Bowen's disease and actinic keratosis are devoid of MMP12. Thus it is thought that expression in transformed epithelial cells correlates with more aggressive histological changes (Kerkela *et al.*, 2000). Keratinocyte derived MMP12 is also found in aggressive vulval SCC (Kerkela *et al.*, 2002). Macrophage derived MMP12 however is more common in well-differentiated vulval tumours and predicts a better clinical outcome (Kerkela *et al.*, 2002). Overall, MMP12 plays a role in cell migration past the epidermal and vascular basement membrane via collagen IV breakdown (Shipley *et al.*, 1996) which might explain its presence in more aggressive cancer subtypes

MMP12 is also found in in granulomatous skin diseases such as sarcoidosis, necrobiosis lipoidica diabeticorum, and granuloma annulare (Vaalamo *et al.*, 1999). It is also expressed by subepithelial macrophages in dermatitis herpetitformis (Salmela *et al.*, 2001) and duodenal ulcers where its upregulation is probably a general response to inflammation and wound healing.

The role of MMP12 in photoageing

Solar Elastosis is the term used to denote tangled degraded elastic fibres, amorphous disorganized tropoelastin and fibrillin with reduced amounts of collagen. In addition, glycosaminoglycans and proteoglycans increase in photodamaged skin, whereas the amount of collagen decreases (Calderone and Fenske, 1995; Yaar and Gilchrest, 2007). It can be subtyped into early and late stages. The early stage is characterised by an accumulation of insoluble disorganised elastin and microfibrillar proteins (fibronectin) (Chen *et al.*, 1986; Lavker and Kligman, 1988), which is clinically evident on the face as a waxy, thickened and furrowed complexion, and the late stage is characterised by degeneration of elastin that appears as lucent areas on electron microscopy and gives a mottled appearance to the dermis. This is clinically associated with a loss of skin elasticity (likely due to sagging) which been shown to be a UVA1 dependent process in hairless mice with a peak at 340nm (Bissett *et al.*, 1987, 1989) whilst wrinkling is a more UVB mediated process.

Adding an elastase to external cultures of elastin (Braverman and Fonferko, 1982) gives the fibres a mottled appearance that is similar to that seen in late solar elastosis. In fact, this effect can be seen in other laboratory studies (Kumakiri *et al.*, 1977) with repeated erythemal doses of UVA, which produces 'zebra bodies' in the dermis, similar to the mottled appearance in late solar elastosis. These changes do not occur after SSR suggesting that elastin breakdown only occurs after UVA irradiation probably via elastase upregulation.

A hypothetical model for UVA1 induced MMP12-associated photoageing is shown below in Figure 5.13.

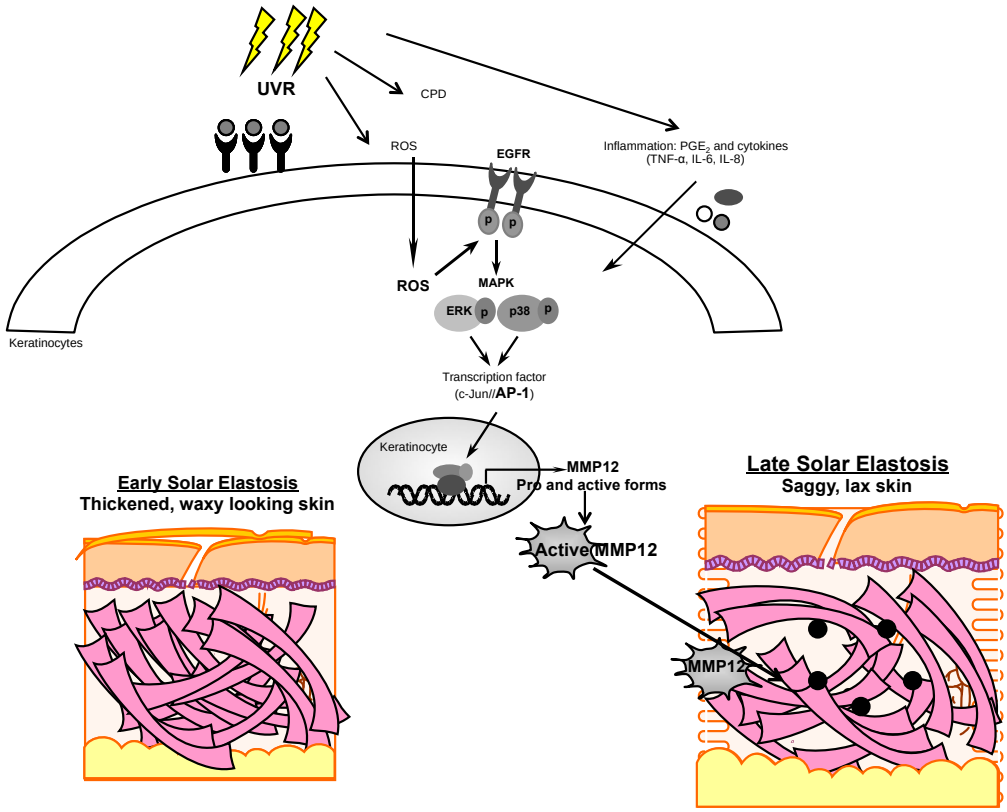


Figure 5.13 Hypothetical model of UVA1 induced late solar elastosis
MMP12 is upregulated by UVA1 at 24h and its breakdown of elastin in the dermis gives the mottled appearance that is seen on electron microscopy which contributes to the appearance of saggy lax skin of late solar elastosis.

Summary

Inflammation, immunosuppression, antioxidant activity are important processes that occur 6h after an acute erythemal dose of UVB or UVA1.

The data in this chapter confirms that UVB plays an important role in photoageing because of its ability to upregulate a range of MMP. We show that MMP12 is a biomarker of UVA exposure and may be a useful endpoint in photoprotection studies. Solar UVA1 induces the expression of MMP12, which degrades elastin and probably contributes to the loss of skin elasticity seen in late solar elastosis. MMP12 may also have a role in photocarcinogenesis, as a marker of skin cancer and disease but possibly also facilitating skin cancer invasion past the basement membrane.

Chapter 6 UVA1 induced cellular and molecular changes in the skin after topical application of DNA repair enzymes: Pilot experiments

Abstract

DNA repair enzymes can enhance CPD repair and thus downstream consequences of the CPD on biological processes can be examined. In this chapter, a pilot experiment showed a reduction of CPD at 6h after a 2MED SSR irradiation in the presence of topical DNA repair enzymes (T4N5 and photolyase). This provides the necessary platform to examine specific biological pathways after UVA1 and UVB irradiation and topical enzyme.

6.1 Introduction

Much of our understanding of the downstream biological responses to DNA damage has come from studies using DNA repair enzymes. The hypothesis that DNA damage is thought to initiate UVR induced immunosuppression is based on the following evidence:

(1) UVR induced suppression of CHS in the South American opossum (*Monodelphis domestica*), whose DNA damage is repaired by a visible light-activated photoreactivating enzyme, was completely prevented by exposing opossum skin to visible light immediately after UVB irradiation (Applegate *et al.*, 1989)

(2) Topical application of T4N5 (bacteriophage T4 endonuclease V, an excision repair enzyme for CPD in DNA) to UVB irradiated mouse skin prevented UVB induced suppression of DTH and CHS responses and induction of suppressor T cells (Kripke *et al.*, 1992)

(3) IL10, which has been shown to be responsible for systemic immunosuppression, is produced by cultured keratinocytes after UVR, but not by keratinocytes pretreated with T4N5, suggesting that UV induced DNA

damage may trigger the production of soluble immunosuppressive mediators like IL10 from keratinocytes (Nishigori *et al.*, 1996). Also CPD affects MMP1 (Dong *et al.*, 2008) and TNFα levels (Walker and Young, 2007).

The first study in this chapter was a pilot study in order to establish a working protocol.

6.2 Materials and methods

The liquid enzymes were a gift from Dr Olivier Doucet, Coty-Lancaster, Monaco. These were ultrasome (liposome containing T4N5 enzyme) and photosome (liposome containing photolyase). We also had empty liposomes as a control group, and 2MED irradiated and unirradiated skin as positive and negative controls. The emission spectrum of the SSR source is shown in Chapter 2 Materials and Methods Figure 2.1.

A pilot experiment with two individuals was performed using 2MED SSR with a view to first demonstrating a decrease in CPD staining after topical application of repair enzymes. Volunteer demographics and MED are shown in the following table:

Study	Skin type	Sex	Age (years)	SSR MED (J/cm^2)
001				
	I	M	24	5.4
002	I	M	24	5.4

Figure 6.1 Volunteer demographics for DNA repair enzyme study

Following 2MED SSR of 4 sites (B, C, D, E), liquid enzyme (~1ml) was placed on buttock skin into stainless steel O-rings that were held on the skin with double sided

tape. Volunteers were asked to remain lying down for 30 minutes after irradiation. Sites were covered and they then returned 6h later for 4mm punch biopsies, which were processed as described in Material and methods.

The irradiation site map is shown below and all biopsies were taken at 6h.

Irradiation Site Map:

A	**B**	**C**	**D**
	E	**F**	**G**

A: Unirradiated control
B: 2 MED SSR (positive control)
C: 2MED SSR+ultrasome
D: 2MED SSR+photosome
E: 2MED+liposome (empty)

6.3 Results

In this pilot study we expect significant CPD formation after 2MED SSR, a decrease after topical enzyme preparations and possibly minimal changes with the empty liposome due to a barrier effect. There are a series of limiting factors in this experiment. As the topical enzyme preparations are liquids, difficulties arise in securing it in place on buttock skin for 6h. Once participants are mobile, there is a possibility that the preparations leaked, merged into each other and contaminated results. Care also had to be taken during application due to the curvature of buttock skin to prevent sites merging. Experimentally this is likely to have occurred with volunteer 001 as we found lowest CPD at site B, which was the positive 2MED SSR control (*data not shown*). Therefore we had to discard all data points with this volunteer. Below is example of staining and CPD intensity quantification for volunteer 002 (Figure 6.2 and Figure 6.3). For the quantification, the intensity of staining from three slides at each site was averaged.

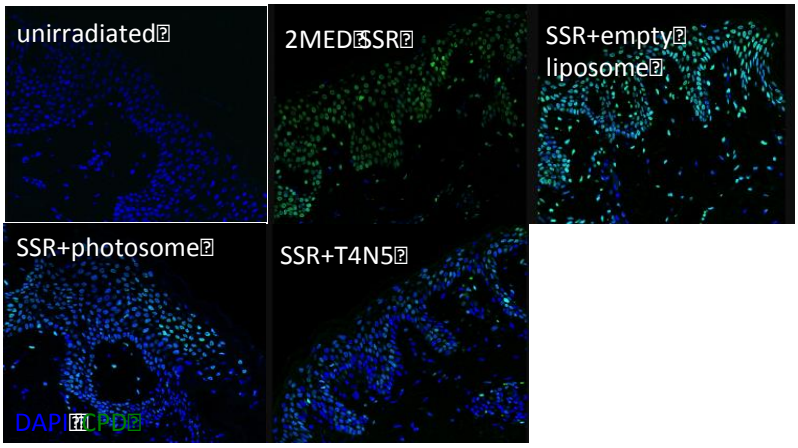


Figure 6.2 Staining for volunteer 002 after application of topical enzymes
(Note that CPD staining is shown as green)

Graph showing CPD (%) remaining compared to the positive control (put at 100%) is shown below. Background staining in the unirradiated control was removed from all sections prior to calculations.

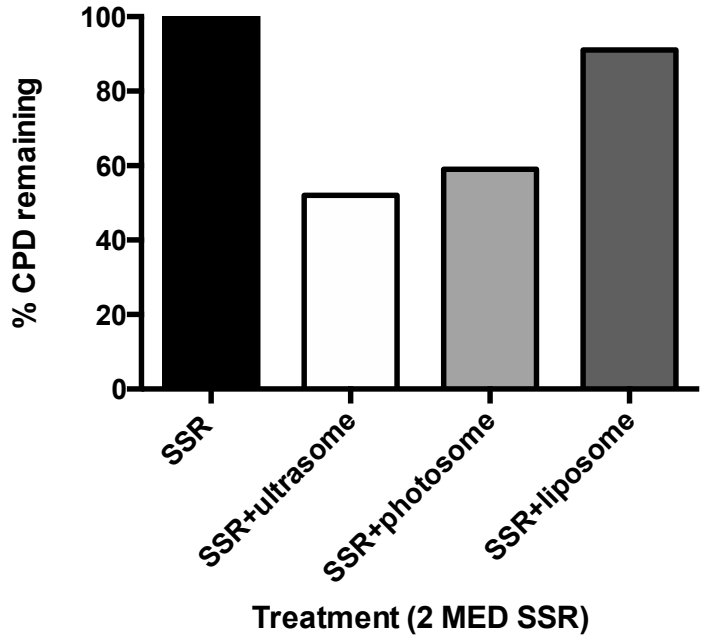


Figure 6.3 CPD remaining as % of positive control
Rapid repair of CPD after SSR following addition of repair enzymes (ultrasome or photosome).

6.4 Discussion

In one individual, the biggest decrease in CPD is found after ultrasome (50% decrease) which contains T4N5, followed by photosome (45% decrease) containing photolyase. This could be that T4N5 is more effective at DNA repair, or that photolyase was not fully activated in our study by the UVA content of SSR. Other studies using topical photolyase found maximum CPD after a 30 minute exposure with 40-45% fewer CPD produced then the positive control 2MED (288mJ/cm^2) irradiation (Stege *et al.*, 2000). Although not directly comparable as we used a different UVR source (with a different UVB content) and our exposures were 10 minutes long we found a similar decrease in CPD after photolyase. There is also a slight decrease in CPD formation after topical liposome, which is likely a variation of normal as n=1. Empty liposomes are lipid soluble and although it is known that oil increases penetration of UVR through it (Diffey, 2002), the empty liposomes are opaque, which reflects visible light and also might change the scattering properties of UVR and affect its penetration. This barrier effect is minimal however and insufficient to produce a CPD protection factor, a concept previously introduced as a measure of reduction of DNA damage after topical products (Young *et al.*, 2000). We see a CPD protection factor of about 2 for photolyase and ultrasome (T4N5).

Summary

There is a need for chemoprevention (Yarosh *et al.*, 2001) especially for high risk patients such as those with genetic predispositions for DNA damage (XP, basal cell naevus syndrome, albinism), organ transplant recipients on immunosuppressive therapy, patients who have received large numbers of PUVA treatments as well as those with many actinic keratosis (Bath-Hextall *et al.*, 2007). This is particularly

important for XP patients where the first skin cancer typically occurs in children younger than 10 years. The disease is disfiguring and the mainstay of treatment is surgery, resulting in many procedures and significant morbidity (pain, recovery time and financial strain). Oral retinoids have been shown to be effective in this patient group yet although effective, large doses are required and beneficial effects are not apparent once the drug is discontinued (Kraemer *et al.*, 1988). Also the side effect profile is unacceptable. Ideally, a chemopreventative agent would inhibit skin cancer with minimal toxicity to the patient and would preferentially affect premalignant or malignant cells leaving normal cells unaffected. Sunscreens are effective against cutaneous SCC and seem to have no effect on BCC (Green *et al.*, 1999; van der Pols *et al.*, 2006) although they have been shown to reduce UVR induced p53 expression (Ananthaswamy *et al.*, 1997) and TT damage (Young *et al.*, 2007). Use with topical DNA repair enzymes (photolyase, endonuclease (T4N5), and 8oxoguanine glycosylase) shows a reduction in CPD but not 8oxodG (Emanuele *et al.*, 2014) compared to sunscreen alone suggesting benefit of a product with dual function of attenuating UVR and enhancing repair of UVR induced CPD.

Chapter 7 General Discussion

7.1 Introduction

30 years ago UVA1 was suggested as a potential UVR source to deliver a 'safe' dark tan as following irradiation, the skin appeared normal on histology with no SBC (Mutzhas *et al.*, 1981; Plewig *et al.*, 1978). Apoptosis (SBC formation in the epidermis) is an established photoprotective skin response, as is tanning where UVA1 is 3 orders less effective than UVB (300nm) at producing new melanin in order to attenuate further UVR and its damaging effects on the skin (Parrish *et al.*, 1982). The majority of UVA1 'tanning' occurs via a redistribution of oxidised melanin within the skin, which is not photoprotective. UVA1 also has a range of immunomodulatory effects and UVA1 phototherapy is used for treatment of certain skin diseases. In this thesis we studied the cellular and molecular effects of UVA1 on the skin primarily focussing on DNA damage, possible mechanisms of carcinogenesis and pathogenesis of UVA1 photoageing, using a biological human model based on skin type I/II individuals (who have the highest susceptibility to UVR induced biological effects).

7.2 Conclusions

Main findings from thesis

1) *UVA1 is probably more carcinogenic than previously thought*. In Chapter 3 we show, for the first time *in vivo* that compared to erythemally equivalent doses of UVB, UVA1 produces 3-4 times fewer CPD and preferentially these are produced in human skin basal epidermis. This is the location of keratinocyte stem cells and melanocytes. The quantity of UVA1 induced CPD increases with skin depth, and beyond 150μm (i.e. in the dermis) they

are found in larger amounts than with UVB. Overall the repair rate of epidermal UVA1 and UVB CPD is the same, however repair in the basal epidermis, including in melanocytes, is extremely slow after UVA1.

2) *We lack data on UVR transmission through the skin in vivo*. Most studies have been done *ex vivo* (Bruls *et al.*, 1984) and have required the chemical separation of the epidermis from the dermis, which is likely to compromise the skin's optical properties. The CPD studies can also be used as an indicator of UVB and UVA1 transmission/exposure in the different layers of the skin. The data suggest that the basal layer and the dermis are particularly susceptible to a wide range of damage induced by UVA1, for example oxidative damage to DNA, membranes and proteins. Also, although we have assessed for TT, which is not mutagenic, some C containing dimers will be formed at these sites which can potentially form C-T transitions if unrepaired.

3) *A lack of UVA1 induced p53 apoptosis*. In Chapter 4 we show that a lack of CPD repair in the basal epidermis is probably due to an upregulation of p63 by UVA1 that contributes to the lack of p53 driven apoptosis and DNA repair in this layer. The fact that this occurs in rapidly dividing epidermal stem cell layer is particularly worrying as it might suggest how a UVA1 CPD is possibly more carcinogenic than a UVB CPD, as they are more likely to persist and be incorporated in the next cell cycle division.

4) *UVA1 and UVB induce an upregulation of genes involved in the same pathways at 6h and 24h*

At 6h we show that both UVA1 and UVB upregulate the immune response (inflammation, apoptosis, immunosuppression and antioxidant activity) through IL17 signalling and at 24h, both sources induce genes in the ECM pathway.

5) *UVA1 induced MMP12 has elastolytic activity*. Chapter 5 demonstrates the identification of a novel UVA1 marker through a series of gene, mRNA and protein studies. Functional work identifies its possible role in photoageing in late solar elastosis. Elastase activity also synergistically plays a role in carcinogenesis. Studies in neutrophil elastase deficient mice showed that they had less 'saggy' skin and did not produce SCC in response to UVB (Starcher *et al.*, 1996). MMP12 is also upregulated by epithelial SCC (Kerkela *et al.*, 2000). Thus, although not identified in this thesis UVA1 induced MMP12 likely also plays a role in human photocarcinogenesis.
6) *Topical application of T4N5 or photolyase reduces CPD after SSR to human skin*. Chapter 6 is a pilot study demonstrating that compared to the positive SSR irradiated control (2 MED) there is enhanced CPD repair 6h following topical photolyase or T4N5 application and SSR.

7.3 Further perspectives

Oxidative DNA damage: location and repair: 8oxoGua is primarily felt to be a UVA induced photoproduct in the skin which is thought to be mutagenic if unrepaired. It was not possible to get 8oxoGua staining to work reliably for this thesis, despite multiple attempts with different protocols, antibody clones, and treated tissue types (frozen, paraffin embedded tissue). This work however, has been

taken over by another PhD student, using the tissue from the DNA damage dose response and time course studies (see Chapter 3). Our work suggests that following UVA1 the basal epidermis is less well protected from CPD damage than expected. Previous work has found similar CPD levels in melanocytes and keratinocytes (Mouret *et al.*, 2012; Young *et al.*, 1998b) but an increased sensitivity of melanocytes to 8oxodG (2.2 fold higher than in keratinocytes) possibly due to its accumulation mediated by melanin oxidation (Wood *et al.*, 2006). It would be useful to assess 8oxodG production in the skin particularly in the basal layer where it is thought to accumulate (Agar *et al.*, 2004). Previous work has also found a lack of 8oxodG and CPD repair in melanocyte cultures (Wang *et al.*, 2010a).

Stem cells: The basal layer, particularly in the deep rete ridges, contains the inter-follicular epidermis (IFE) stem cells (Blanpain and Fuchs, 2006; Kaur, 2006), which work towards tissue renewal. Other stem cells include the melanocyte stem cells, which can be recruited during wound healing to repair the epidermis. It would be important to look at UVA1 induced DNA damage and repair in stem cells in comparison to erythemally equivalent doses of UVB as UVR induced apoptosis of stem cells in the basal layer and hair bulge is postulated to result in epidermal atrophy, slow wound healing, and depigmented pseudoscars, whereas the greater melanin production observed in senescent melanocytes in response to trauma (Bandyopadhyay and Medrano, 2000) may be responsible for ‘‘bronzing,’’ the permanent ‘‘tan’’ observed in photoaged skin of some darker-skinned individuals.

Gene array studies: We anticipated some clear differences between UVB and UVA1 because of reported differences in mechanisms of action but overall this did

not seem to be the case. The current "dogma" is that UVB effects are caused by direct damage to chromophores and that UVA1 effects are indirect via ROS. We suggest that this may be too simple, especially as we show that UVB also induces oxidative stress. We also suggest that even though pathway responses in the skin following UVB and UVA1 are broadly similar, individual gene expression changes in immunomodulation, apoptosis, antioxidant pathways and p63 contribute to the distinct differences with UVB and UVA1. It is possible that indirect or direct effect may depend on UVR dose and that thresholds may vary from gene to gene.

There are a host of other processes not examined in detail in this thesis including DNA repair (BER, NER) genes and those for epidermal differentiation. It would be important to study these further for any source specific differences.

Our protein validation study was proof of concept work in 3 skin type I/II of similar photosensitivity, where we demonstrated MMP12 protein induction and elastase activity by UVA1. Our results could be expanded in 3 further individuals to increase robustness of the data.

Repeated sub-erythemal exposure: Although our UVA1 doses were single (low to high) dose UVA1 (12.5-148J/cm^2), we have made conclusions on the potential chronic effects in the skin. An obvious extension to the work would be to examine the skin after multiple daily suberythemal UVA1 doses with reference to CPD induction and repair the basal epidermis. MMP1 induction in human skin after multiple UVA1 doses has recently been studied and shown to occur predominantly in the dermis without a concomitant increase in collagen synthesis (type 1 collagen fibrils) (Wang *et al.*, 2013). This is probably a more realistic interpretation of

chronic UVA1 exposure, either via high dose UVA1 tanning lamps or chronic UVA exposure using predominantly UVB sunscreens. MMP1 and MMP12 also accumulate in the dermis in photoaged skin (Chung *et al.*, 2002; Quan *et al.*, 2013; Quan *et al.*, 2009; Wang *et al.*, 2013). A new study giving multiple erythemally equivalent doses of UVA1 and UVB would be a unique opportunity to assess the upregulation and location of MMP1 and MMP12 and might help determine contribution of each source to chronic UVR induced damage. MMP12 is highly inducible, its mRNA is upregulated ~20-35 fold at 6h and 24h after UVA1, and we feel that also studying dose and repair kinetics would give us a deeper understanding of the nature of this enzyme. Previous work shows that MMP12 mRNA reaches a 4-fold maximum 48h post heat exposure (90minutes at 40-42°C) (Chen *et al.*, 2005) thus not only would it be useful to extend our time points but also assess contribution from the infrared emitted from our UVA1 source.

Role of CPD in biological responses: Finally, preliminary evidence of CPD removal using topical T4N5 and photolyase after SSR has been demonstrated in Chapter 6. Expansion of the number of volunteers was not possible in the time span of the thesis, but CPD repair enhancement technology allows the determination of the biological consequences of the CPD. This has been done *in vitro*, in animal studies and in human skin. In a further set of 5 individuals it would be important to assess downstream cellular endpoints as examined in Chapter 5; for example inflammation (IL1, IL6, IL8 mRNA expression), antioxidant activity (FOSL1, PTGS2, HO1 mRNA expression) as well as immunosuppression (IL10 mRNA expression) to give an indication of the nature of the processes triggered by UVA1 ROS and UVB/UVA1 CPD induction.

7.3.1 Impact to human health

Phototherapy

There are a lot of data on the long-term risks of PUVA and UVB phototherapy. Most authors report that patients with ≥ 200 PUVA treatments have a 10–30 fold increased risk of developing SCC and up to 5 fold increase of BCC (Bruynzeel *et al.*, 1991; Lindelof *et al.*, 1991; Stern *et al.*, 1998). There is also a 286 fold increase in genital SCC following PUVA treatment. The risk of MM is approximately 5 fold increased in patients who have received > 250 treatments (Stern *et al.*, 1997).

In the case of broadband UVB therapy, a matched analysis revealed that exposure to >300 treatments increased the risk of genital SCC by about 4 fold (Stern, 1990), which is different to early studies that reported no significant cancer risk (Maughan *et al.*, 1980; Studniberg and Weller, 1993). A recent study in 4000 patients treated with NBUVB showed no increased risk of BCC, SCC or MM after a mean of 29 treatments, with approximately 10% having greater than 100 treatments (Hearn *et al.*, 2008). However these results need to be interpreted with caution as most patients only had 2 cycles of phototherapy. Concomitant PUVA therapy increases the risk of BCC. Based on these studies, most photodermatology centres set an upper limit of 200 treatments of PUVA and 300 treatments for NBUVB phototherapy and protection to eyes and genitals.

Currently there are no published data on the risks of NMSC and MM after UVA1 therapy. This thesis provides evidence that UVA1 is potentially mutagenic and carcinogenic, and contributory to photoageing. This thesis also provides an objective assessment for likely MMP1 depth effect in the skin as at erythemal doses

we show more induction by UVA1 of dermal MMP1 compared to UVB (300nm) (Chapter 5) after 150μm. Our findings of UVA1 induced MMP12 with elastase specificity is novel and might explain the contribution of UVA1 in late solar elastosis. Current anti-ageing strategies involve the use of topical antioxidants and DNA repair enzymes, and strategies with the use of topical anti-MMP have not yet been targeted although MMP affect a large number of metabolic processes and its use in the treatment of cancer has been controversial (Zucker and Cao, 2009). Topical anti-MMP12 encapsulated in liposomes might be a future strategy in patients exhibiting late stages of photoageing. It is also important to note that combating photoageing is likely to also indirectly combat photocarcinogenesis.

Sunbed use

UVR is classified as a carcinogen (El Ghissassi *et al*., 2009) and there is a 75% increase in melanoma risk (IARC, 2007) when first exposure to a sunbed is before 35 years of age. 9 out of 10 sunlamps emit above the legislation limits for irradiance (Tierney *et al*., 2013) and in the UK there is a law prohibiting sunbed use by those under 18 of age. The data in this thesis provide additional support for these restrictions, given that the major output of sunbeds is in the UVA1 spectral region.

Photoprotection

The role of UVA in MM remains controversial. Some epidemiological analyses, based on differential latitude gradients for UVB and UVA, provide evidence for an important role of UVA (Moan *et al.*, 2012a). It has been suggested that the lack of good UVA protection in early sunscreens (Diffey and Farr, 1989) may explain the

positive association between MM and sunscreen use reported in some studies (Autier *et al.*, 2011; Gorham *et al.*, 2007; Westerdahl *et al.*, 1995). This could be due to increased sunshine exposure particularly at doses below the sunburn threshold (Autier *et al.*, 1999; Autier *et al.*, 2000; Young, 2000) and whilst this has been extensively debated (Huncharek and Kupelnick, 2002) a recent RCT showed a significant decrease in the incidence of melanoma with the regular use of a broad band SPF15 sunscreen (Green *et al.*, 2011). Lack of UVA protection may also be a factor in poor immunoprotection by sunscreens (see Introduction for discussion on CPD and UVR induced immunosuppression) (Kelly *et al.*, 2003; Poon *et al.*, 2003; Ullrich *et al.*, 1999), which if verified, would support enhanced UVA protection by sunscreens.

There has been a recent trend for greater UVA1 protection in sunscreens and several regulatory authorities now require minimum levels of UVA protection (Osterwalder *et al.*, 2014). The consequence of this is reduced UVB protection for a given SPF. In general, this trend has not had a biological basis, and indeed UVA protection has been widely assessed by PPD, which has no known biological significance, or by arbitrary spectral properties (e.g. critical wavelength or Boots star rating). In the US, the FDA regulates sunscreens as drugs and it is more difficult than Europe (where sunscreens are treated as cosmetics) to obtain approval for new filters. One consequence of this is a lack of sunscreens with good UVA1 protection. The data generated in this thesis support enhanced UVA1 protection by sunscreens especially in relation to damage to the basal layer and the dermis and also for immunoprotection. Furthermore, UVA1 exposure is much less dependent on

latitude, season and time of day than UVB exposure. Thus, UVA1 protection may be more important than UVB protection under certain conditions (particularly at the end of day and in temperate latitudes), but this remains to be established.

Appendices

Appendix A: Publications produced from work contained in this thesis:

Tewari A, Grys K, Kollet J, Sarkany R, Young AR. Up regulation of MMP12 and Its Activity by UVA1 in Human Skin: Potential Implications for Photoaging. *J Invest Dermatol.* 2014 134 (10) 2598-609

Tewari A, Grage MM, Harrison GI, Sarkany R, Young AR. UVA1 is skin deep: molecular and clinical implications. *Photochem Photobiol Sci* 2013;**12**(1):95-103

Tewari A, Lahmann C, Sarkany R, Bergemann J, Young AR. Human erythema and matrix metalloproteinase-1 mRNA induction, in vivo, share an action spectrum which suggests common chromophores. *Photochem Photobiol Sci* 2012;**11**(1):216-23

Tewari A, Sarkany RP, Young AR. UVA1 induces cyclobutane pyrimidine dimers but not 6-4 photoproducts in human skin in vivo. *J Invest Dermatol*. 2012;**132**(2) :394-400

Appendix B: Publications demonstrating knowledge of photodermatology

Tewari A, Fassihi H, McGibbon D, Robson A, Sarkany R. A case of extensive hyaline deposition in facial skin caused by erythropoietic protoporphyria. *Br J Dermatol.* 2014 Aug;171(2):412-4

Tewari A, Marsden J, Naik H, Benton EC, Sarkany R. Oral cholestyramine is not an effective treatment for uncomplicated erythropoietic protoporphyria. *J Am Acad Dermatol.* 2012;**67**(6):1383-4

Tewari A, Garibaldinos T, Lai-Cheong J, Groves R, Sarkany R, Branislav Novakovic L. Successful treatment of microstomia with UVA1 phototherapy in systemic sclerosis. *Photodermatol Photoimmunol Photomed*. 2011;**27**(2):113-4

Presentations arising from work in this thesis

Tewari A: MMP12 is induced by UVA1 but not UVB British Association of Dermatology (2013), Liverpool, UK

Tewari A: UVA1 induces key gene pathways associated with inflammation, antioxidant activity, apoptosis, immunosuppression and extracellular matrix remodelling British Association of Dermatology (2012) Birmingham, UK

Tewari A: UVA1 induces distinct and different epidermal changes compared to UVB associated with p53, p63 and keratin differentiation American Society of Photobiology (2012), Montreal, Canada

Tewari A: Does the mechanism of UVA1 induced TT dimers in the epidermis *in vivo* determine their distribution? British Association of Dermatology (2011), London, UK

Tewari A: UVA1 induced epidermal and dermal thymine dimers *in vivo* European Society of Photobiology (2011) Geneva Switzerland

Tewari A: UVA1 induces key molecular events associated with inflammation, response to oxidative stress and immunosuppression *in vivo* in humans European Society of Photobiology (2011) Geneva Switzerland

Poster presentations arising from work in this thesis

Tewari A, Grys K, Sarkany R, Young AR MMP12 is upregulated by UVA1: potential implications for photoageing (2014) British Society of Investigative Dermatology, Newcastle, UK

Tewari A, Grys K, Sarkany R, Young AR UVA1 is a specific biomarker in human skin in vivo International Investigative Dermatology (2013), Edinburgh UK

Tewari A, Grys K, Sarkany R, Young AR UVA1 induces key molecular events associated with inflammation, response to oxidative stress and immunosuppression *in vivo* in humans European Society of Dermatological Research (2011), Barcelona, Spain

Tewari A, Sarkany R, Young AR UVA1 induces Cyclobutane Pyrimidine Dimers *in vivo* British Society of Investigative Dermatology (2011), Manchester.

Appendix C Heat map showing gene expression profiles of UVA1 and UVB at 6h (D, E) and 24h (G, F) for selected genes with antioxidant activity

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Upregulation of MMP12 and Its Activity by UVA1 in Human Skin: Potential Implications for Photoaging

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UVA1 constitutes around 75% of the terrestrial UV radiation, and most of the output of artificial tanning sources. However, the molecular effects of UVA1 in human skin *in vivo* are surprisingly poorly understood. We have examined time-dependent whole-genome expression, along with mRNA and protein changes in the skin after one minimal erythema dose of spectrally pure UVA1 (50 J cm^{-2}) and 300 nm UVB (30 mJ cm^{-2}). After 24 hours, the genes induced to the greatest extent were those involved in extracellular matrix remodeling with both UVA1 ($P = 5.5e-7$) and UVB ($P = 2.9e-22$). UVA1 and UVB caused different effects on matrix metalloproteinase (MMP) expression: UVB induced MMP1, MMP3, and MMP10 mRNA at 24 hours to a much greater extent than UVA1. MMP12 induction by UVA1 at 6 hours is marked and much greater than that by UVB. We have found that MMP12 mRNA induction by UVA1 resulted in expression of MMP12 protein, which is functional as an elastase. This induction of elastase activity did not occur with UVB. We hypothesize that the UVA1 induction of MMP12 mediates some of its photoaging effects, particularly by contributing to elastin degeneration in late solar elastosis. MMP12 is a good marker of UVA1 exposure.

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INTRODUCTION

Terrestrial solar UV radiation (UVR) comprises <5% UVB (~295–315 nm) and >95% UVA (315–400 nm), the majority (~75%) of which is UVA1 (340–400 nm). UVA1 also makes up most (~80%) of the spectral output of sunbeds and is used at high doses as a specialist form of phototherapy (Kerr *et al.*, 2012). However, the acute effects of UVA1 *in vivo*, including its effects on gene expression, are much less well understood than those of UVB (Enk *et al.*, 2004; Enk *et al.*, 2006). As it is now clear that UVA1 is biologically active and mutagenic in human skin *in vivo* (Mouret *et al.*, 2006; Tewari *et al.*, 2012), it is clinically important to understand the biological effects of UVA1 in the skin. Previous studies on the effects of UVA1 on whole-genome expression *in vivo* have been limited and have primarily focused on understanding mechanisms of pigmentation (Choi *et al.*, 2010).

The hallmarks of long-term exposure to solar UVR are photoaging (Yaar and Gilchrest, 2007) and photocarcinogenesis (Sage *et al.*, 1996). Photoaging is characterized by the induction of extracellular matrix-degrading proteolytic enzymes (matrix metalloproteinases, MMPs) without a parallel induction of inhibitors of proteolysis (tissue inhibitor of metalloproteinases). The resulting pathological remodeling process involves the degradation of collagen and the accumulation of abnormal elastin in the superficial dermis, resulting in the characteristic changes of solar elastosis (Chen *et al.*, 1986; Uitto, 2008). Previous studies with UVR sources rich in UVB showed induction of MMP1, MMP3, and MMP9 mRNA (Brenneisen *et al.*, 1996; Fisher *et al.*, 1996; Fisher *et al.*, 1997) in human skin *in vivo*. UVA sources have also been reported to induce MMP1 expression in fibroblasts (Scharffetter *et al.*, 1991; Herrmann *et al.*, 1993). There are few data on the effects of spectrally pure UVA1 on MMPs in human skin *in vivo* (Wang *et al.*, 2013).

MMP activity is required both in normal physiological processes such as wound healing and angiogenesis (Chakraborti *et al.*, 2003) and in the pathological tissue destruction that occurs in chronic wounds, dermal photoaging, bullous skin disease, cancer invasion, and metastasis (Kerkela and Saarialho-Kere, 2003). The mechanisms by which UVR induces MMPs are poorly understood. Some studies have reported that this occurs via the generation of reactive oxygen species (Scharffetter-Kochanek *et al.*, 1993; Fisher *et al.*, 2009), whereas others have suggested that DNA is a major chromophore and that MMPs are triggered by the formation of cyclobutane pyrimidine dimers (CPDs) (Dong *et al.*, 2008). MMPs may also be induced by mediators such as tumor necrosis factor α (Steenport *et al.*, 2009), which is also readily induced in the skin by UVR, probably via the induction of

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Abbreviations: MMP1, matrix metalloproteinase 1; MMP12, matrix metalloproteinase 12; UVA1, ultraviolet A1 (340–400 nm)

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CPDs (Walker and Young, 2007) and IL6 (Wlaschek *et al.*, 1994).

It is widely considered that UVA has a larger role than UVB in photoaging, both because of the deeper penetration of UVA into the dermis (Bruls *et al.*, 1984) and because of the sensitivity of fibroblasts to UVA-induced MMPs (Scharffetter *et al.*, 1991; Herrmann *et al.*, 1993). There are inherent problems with previous studies that relate to spectral purity of the source of UVR. UVB sources often also emit a considerable amount of UVA and it is not possible to attribute an effect to UVB without knowledge of the action spectrum of the end point under investigation. Even minor contamination of a nominal UVA source with very small amounts of UVB may give misleading results because <1% UVB contamination can be responsible for most of a given effect, e.g., DNA damage (Woollons *et al.*, 1999). Also, UVA2 (315–340 nm) and UVA1 are biologically and mechanistically different. Thus, we have concentrated our studies on the biological effects of spectrally pure UVB (300 nm) and UVA1. In a recent work, we reported that solar UVB exposure was probably the most important factor in MMP1 induction from an environmentally relevant perspective (Tewari *et al.*, 2012). In this study, we compare the effects *in vivo* of erythemally equivalent and biologically relevant doses of spectrally pure UVA1 and UVB on whole genome expression, mRNA, and protein and enzyme activity of the most significantly enriched pathway at 24 hours (extracellular matrix remodeling). We have used this approach because erythema is the widely used end point in clinical and experimental photodermatology. We chose 300 nm because it is in the region of the peaks of the action spectra both for erythema and for CPD induction in human skin *in vivo* (Young *et al.*, 1998) and its photobiological effects are likely to be mechanistically different from UVA1. Furthermore, erythemal exposure, quantified by the standard erythema dose, is increasingly used as a measure of UVR exposure in clinical and epidemiological studies.

RESULTS

Extracellular matrix remodeling genes are induced to a greater extent than other pathways, both by UVA1 and by UVB 24 hours after exposure.

We used Genego Metacore v7 on our microarray data to identify upregulated pathways at 6 and 24 hours using the pooled intensities ($n=9$ for UVA1 and $n=5$ for UVB) of upregulated genes compared with each individual's nonirradiated control ($P<0.05$, fold change $\geqslant 2$). At 6 hours, the most significantly enriched pathway was inflammation through Th17 signaling for erythemally equivalent doses of UVA1 ($P=1.16e-6$) and UVB ($P=2.1e-4$). At 24 hours, the most significantly enriched pathway was extracellular matrix remodeling for UVA1 ($P=5.5e-7$) and UVB ($P=2.9e-22$). Many other groups of genes were induced to lesser extents but this paper will focus on the MMPs, given their striking degree of induction.

Whole-skin microarray analysis at 6 (Figure 1a) and 24 hours (Figure 1b) after exposure indicated that genes encoding MMP1, MMP3, MMP9, MMP10, and MMP12 are induced predominantly at 24 hours. MMP1, MMP3, and MMP10 (10–550-fold, $P\leqslant 0.05$, adjusted $P\leqslant 0.3$) were induced to a greater extent by UVB, whereas UVA1 induced a 15-fold increase in MMP12 gene expression at 6 hours ($P\leqslant 0.05$, adjusted $P\leqslant 0.3$) that increased to around 30-fold at 24 hours ($P\leqslant 0.05$, adjusted $P\leqslant 0.3$).

Key UVA1 and UVB mRNA expression differences

Specific genes assessed by reverse transcription quantitative PCR (qPCR) at 6 and 24 hours are shown in Figure 1c–h. At 24 hours, UVB was more effective than UVA1 at inducing MMP1 ($P=0.0062$, UVB/UVA1 fold difference 13.2) (Figure 1c), MMP3 ($P=0.0016$, UVB/UVA1 fold difference 38.7) (Figure 1d), and MMP10 ($P=0.028$, UVB/UVA1 fold difference 27.1) (Figure 1e). Both UVA1 and UVB induce MMP9 mRNA to an equal extent (at 6 and 24 hours post exposure (Figure 1f); $P=0.33$ at 6 hours, $P=0.12$ at 24 hours). UVA1 was more effective than UVB at inducing MMP12 mRNA at 6 ($P=0.02$, UVA1/UVB fold difference 19.2) and 24 hours ($P=0.22$, UVA1/UVB fold difference 8.26). However, the large interindividual variation at 24 hours means that the difference is not significant (Figure 1g). UVA1 did not induce MMP3 or MMP10.

UVR-induced MMP1 protein and its activity in the epidermis

Typical MMP1 and MMP12 protein staining (red fluorescence) and enzyme activity (*in situ* zymography shown by green fluorescence) are shown in Figure 2a and b, respectively. UVB and UVA1 induce MMP1 protein (at 10 and 24 hours (Figure 2c)) to a similar extent. Enzyme activity is preferentially induced by UVB at 24 hours compared with UVA1 ($P=0.031$) (Figure 2d). DQ collagen type I is a substrate for MMP1, MMP2, MMP3, MMP9, and MMP13 (Yan and Blomme, 2003). Figure 3a shows that MMP2 protein was not induced by either spectrum at 24 hours. However, as shown in Figure 3b, UVB induced an increase in MMP9 protein at 24 hours.

UVA1 preferentially induces MMP12

MMP12 protein is predominantly formed by UVA1 at 24 hours ($P=0.04$) (Figure 2e). A lesser degree of induction of MMP12 protein is seen with UVB at 10 and 24 hours, despite the absence of MMP12 mRNA induction by UVB at 6 and 24 hours. There is significantly ($P=0.027$) more MMP12 activity at 10 hours after UVA1 exposure than after UVB (Figure 2f).

Location of MMP1 and MMP12 proteins within the epidermis

Figure 2g and h show that MMP1 protein is mainly induced in the upper epidermis (upper versus lower epidermis UVB $P=0.005$ and UVA1 $P=0.01$), whereas MMP12 is equally induced throughout the epidermis (Figure 2i and j) (upper vs. lower epidermis UVB $P=0.20$, UVA1 $P=0.42$).

UVR-induced MMPs and their activity in the dermis

Expression of MMP1 and MMP12 proteins in the dermis are higher with UVA1 than UVB (Figure 4a and c), but there are no spectral differences in the enzyme activities (Figure 4b and d). Overall, both MMP protein and enzyme activity are higher in the epidermis than in the dermis. Figure 5a shows

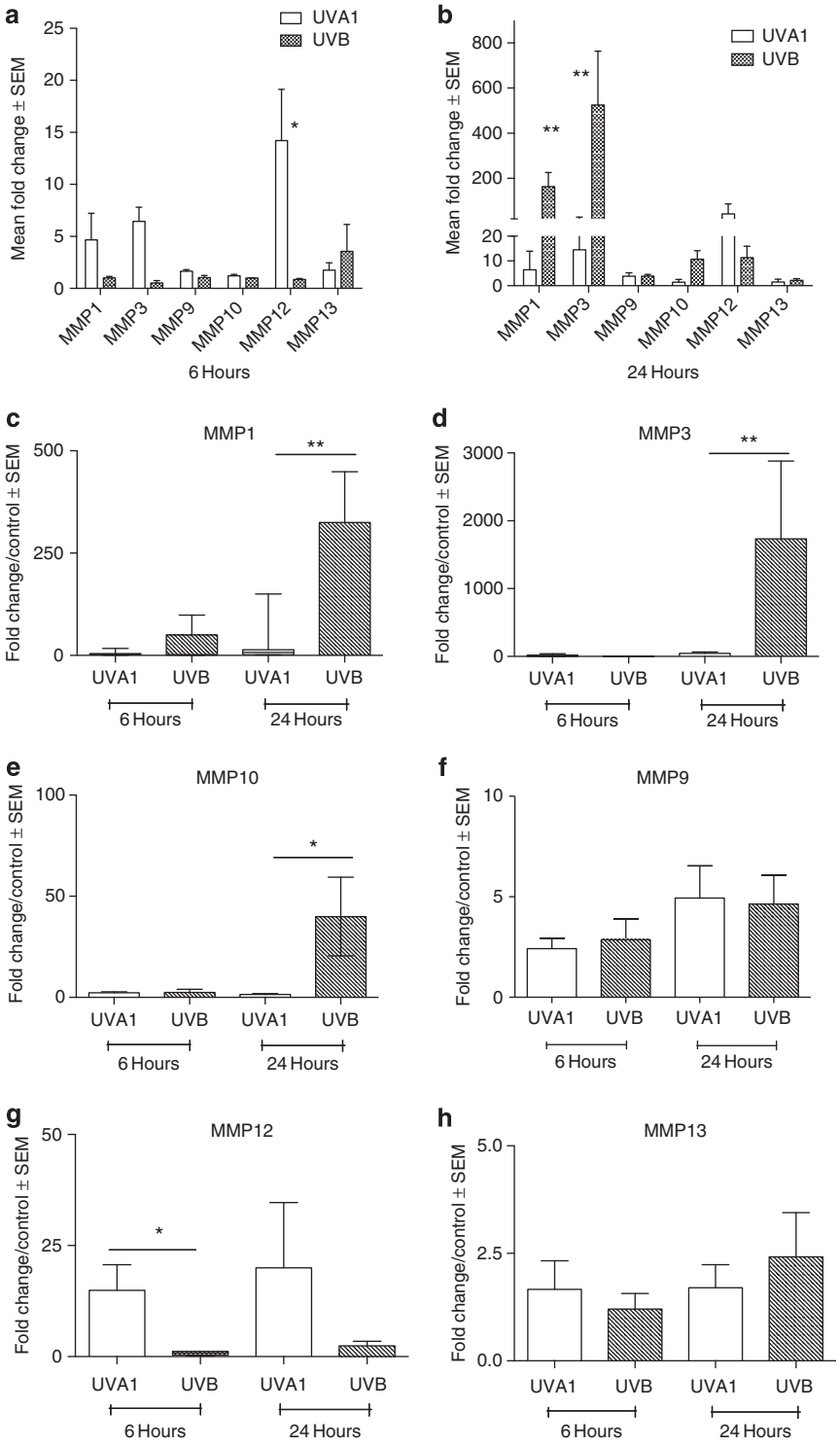

Figure 1. Key UVA1 and UVB gene expression differences in extracellular matrix remodeling: RNA changes. Nine skin type I/II participants for UVA1 and five skin type I/II participants for UVB, RNA extracted and converted to cRNA, and hybridized to Agilent 4 × 44 K microarray chips. Normalized gene expression ratios ($\log_2$) compared with each individual's nonirradiated control at (**a**) 6 hours and (**b**) 24 hours were used for calculations. The individual values were plotted ($P \leqslant 0.05$, adjusted $P \leqslant 0.3$). Validation with reverse transcriptase (RT) quantitative (PCR) qPCR (ΔΔCT method) using Taqman probes and GAPDH housekeeping gene to produce a relative fold change: (**c**) MMP1, (**d**) MMP3, (**e**) MMP10, (**f**) MMP9, (**g**) MMP12, (**h**) MMP13. $*P \leqslant 0.05$, $**P \leqslant 0.01$. Microarray and qPCR were performed on samples from all nine volunteers.

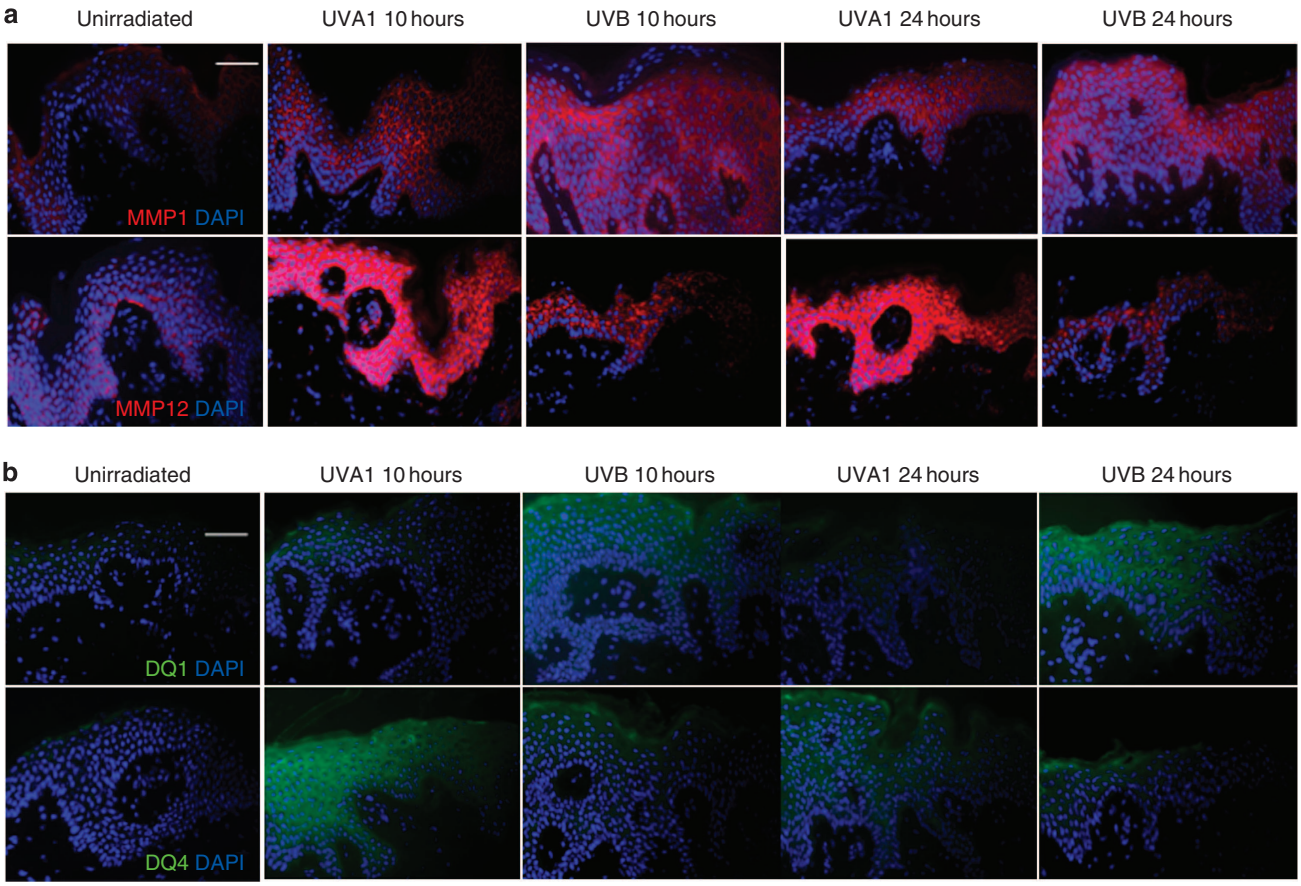

Figure 2. UVA1 induces more epidermal matrix metalloproteinase (MMP)12 protein (immunofluorescence) and enzyme activity (*in situ* zymography) than UVB. MMP1 and MMP12 protein (antibodies from Abcam) immunofluorescence in one representative individual detected by (**a**) alexafluor 555 (red fluorescence) for 30 mJ cm^{-2} UVB (~1 minimal erythema dose (MED)) at 10 and 24 hours and 50 J cm^{-2} (~1MED) of UVA1 at 10 and 24 hours and (**b**) DQ collagen type I (DQ1), substrate for MMP1, MMP2, and MMP9, and DQ collagen type IV (DQ4), substrate for MMP2, MMP7, MMP9, MMP12, and MMP25, were used to detect enzyme activity via fluorescein (green fluorescence), quantification of (**c**) epidermal MMP1, (**d**) epidermal DQ1, (**e**) epidermal MMP12, and (**f**) epidermal DQ4. Distribution of MMP1 and MMP12 within the epidermis, (**g**) UVB-induced MMP1, (**h**) UVA1-induced MMP1, (**i**) UVB-induced MMP12, and (**j**) UVA1-induced MMP12. *$P \leqslant 0.05$, **$P \leqslant 0.01$; $n = 3$. Scale bar = 50 μm.

that UVA1, but not UVB, induces elastin breakdown in the epidermal region.

Macrophages are a potential source of UVA1-induced MMP12

Figure 5b shows significant depletion of dermal macrophages (CD68$^+$) 24 hours after UVB exposure. In contrast, UVA1 had no effect on dermal macrophage numbers ($P = 0.21$) compared with nonirradiated controls.

DISCUSSION

We have compared UVR-induced MMPs using spectrally pure UVB (300 nm) and UVA1 on the same individuals, which is likely to reduce the impact of interpersonal variation. Our UVA1 doses are physiologically relevant; 50 J cm^{-2} would be a typical dose received from a 2.5 hour exposure to the tropical Australian sun (19 °S) (Bernhard *et al.*, 1997) and higher doses are regularly given for treatment of sclerosing skin conditions (Kerr *et al.*, 2012).

Baseline expression of MMPs is usually low in human tissue, including skin. Our gene array data (supported by qPCR) show that erythemally equivalent doses of UVB and UVA1 induce many MMPs. There was no increase in tissue inhibitor of metalloproteinases 1–4 with either spectrum assessed by microarray (data not shown). For most MMPs, induction by UVB peaked at 24 hours, although some expression is seen at 6 hours with some MMPs. MMP12 gene and mRNA expression by UVA1 is seen at 6 hours but primarily at 24 hours. The most striking fold increases with UVB were for MMP1 and MMP3 with array and qPCR technology. There was also an impressive increase of MMP10 mRNA with qPCR. Neither UVB nor UVA1 had any effect on MMP2 assessed by microarray, mRNA (data not shown), and protein. The most striking effect of UVA1 was on MMP12, as assessed by the three techniques. In contrast, UVB had little effect on MMP12 expression. There was modest or no evidence of any UVA1 induction of MMP3 or MMP10 by qPCR. MMP12 mRNA expression has been reported in human skin *in vivo* at 16 and 24 hours by others (Chung *et al.*, 2002), after a 2 minimal erythema dose (MED) exposure from a broad-spectrum UVB–UVA source. In another study, no MMP12 mRNA was

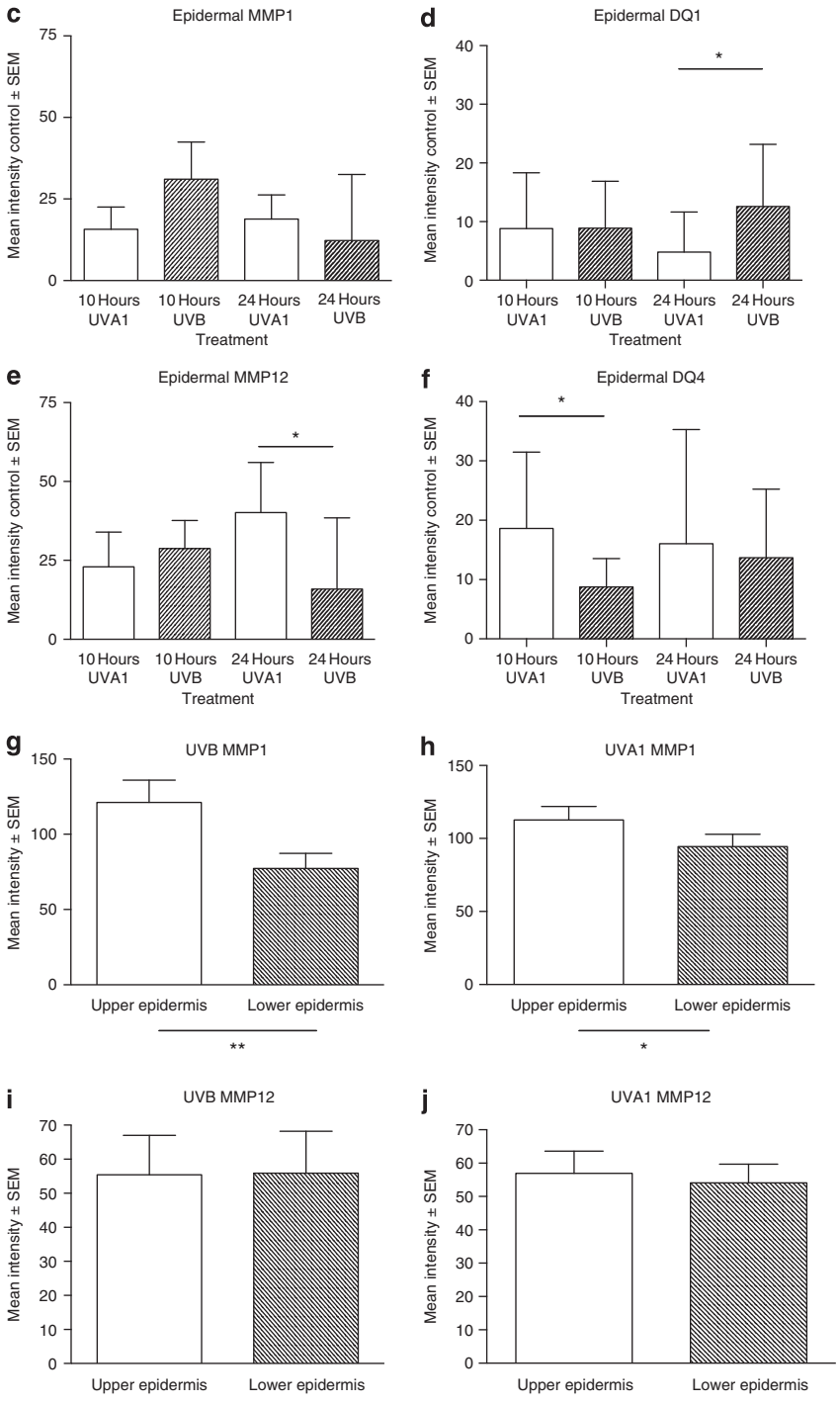

Figure 2. Continued.

detected after exposure to UVA1 or broad-spectrum UVR (UVB with UVA) for three consecutive days (Saarialho-Kere *et al.*, 1999).

Recently, it was shown that 40 J cm^{-2} UVA1 in human skin *in vivo* increased MMP1 and MMP3 mRNA by ~80- and 50-fold, respectively, at 24 hours (Wang *et al.*, 2013). These data are comparable to the responses we see to 50 J cm^{-2} UVA1 (especially for MMP3), which are 25- and 45-fold compared to nonirradiated control tissue for MMP1 and MMP3, respectively (see Figure 1c and d), given the error range in both data sets. Repeated (4 ×) low-dose UVA1 on human skin *in vivo* results in an accumulation of MMP1 and MMP3 mRNA expression (Wang *et al,* 2013).

Our protein studies show that UVR-induced MMPs are predominantly expressed in the epidermis, which is expected because of their role in epithelial tissue homeostasis after UVR

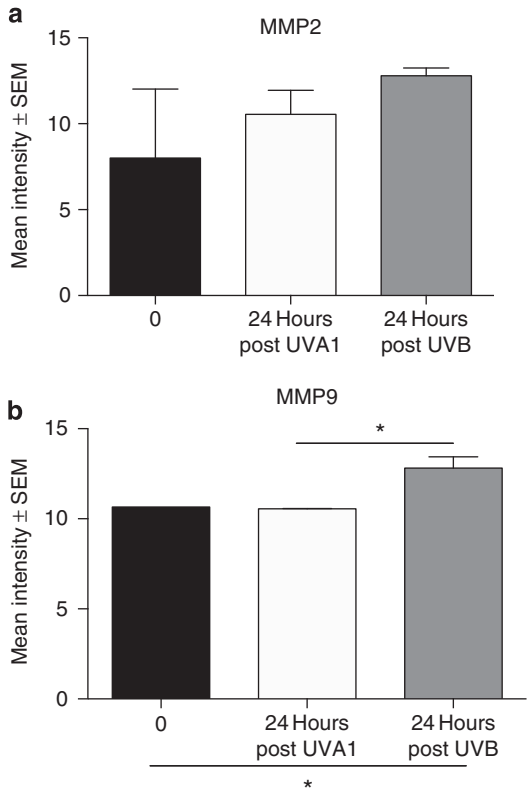

Figure 3. Effects of UVA1 and UVB on matrix metalloproteinase (MMP)2 and MMP9 expression in the epidermis. Using immunofluorescence there was (**a**) no differential MMP2 expression at 24 hours following 1 minimal erythema dose of UVA1 and UVB; (**b**) UVB induces significantly more MMP9 than UVA1 at 24 h. *$P \leqslant 0.05$; $n = 3$.

injury. Other studies support this observation (MMP1, MMP3, and MMP9 mRNA and MMP1 activity) after exposure to solar-simulating radiation (Quan *et al*, 2009). However, we believe that our data demonstrate MMP1 protein/activity by spectrally pure UVB and UVA1.

There are eight elastases in human skin (Liang *et al.*, 2006), of which human macrophage elastase (HME) or MMP12 (Shapiro, 1998; Shapiro *et al.*, 1993), and neutrophil-derived (neutrophil elastase (NE)) are induced by UVR (Lee *et al.*, 2008). NE has long-term photobiological significance, because NE-deficient mice are resistant to photoaging (Starcher and Conrad, 1995; Takeuchi *et al.*, 2010) and squamous cell carcinoma formation (Starcher *et al.*, 1996) after exposure to a source containing 10% UVB and 90% UVA. This suggests that elastase may be linked to these two long-term consequences of UVR exposure.

MMP12 protein has been detected in ''a few stromal fibroblast/macrophage-like cells'' after exposure to UVA1 (Saarialho-Kere *et al.*, 1999). A modest induction of dermal fibroblast MMP12 (but not in macrophages) has also been reported in human skin *in vivo* after a 2 MED exposure from a broad-spectrum UVB–UVA source (Chung *et al.*, 2002; Saarialho-Kere *et al.*, 1999). UVB induced significantly greater ($P = 0.02$) depletion of papillary dermal macrophages than did UVA1 (Figure 5b). Thus MMP12 could be explained by the larger presence of activated macrophages in the dermis after UVA1. MMP12-negative macrophages cannot penetrate the dermal/epidermal junction (Shipley *et al.*, 1996), and we found no evidence of epidermal macrophages. We did not stain for NE, but dermal neutrophil infiltration (and likely NE release) is a predominantly UVB-driven process (Lee *et al.*, 2008) and is therefore unlikely to explain our UVA1 data. MMP12 is also produced by activated T cells (Hughes *et al.*, 1998), transformed keratinocytes, and keratinocyte-derived tumors (Kerkela *et al.*, 2000). Given that we found MMP12 primarily in the epidermis, irrespective of spectrum, we suggest that it is mainly derived from normal keratinocytes. However, we cannot exclude the possibility that some MMP12 may be derived from dermal cells such as fibroblasts and macrophages.

UVB and UVA1 induced the degradation of DQ collagen type I. The assessment of specific MMP function by *in situ* zymography is complicated by cross-reactivity. DQ collagen type I is primarily a substrate for MMP1 but is also degraded by MMP2, MMP3, MMP9, and MMP13 (Yan and Blomme, 2003). There is no UVR induction of MMP2 mRNA or protein (Figure 3a) at 24 hours but there is a small (~20%) significant increase in MMP9 protein by UVB (Figure 3b). This has been previously reported (as 92 kd gelatinase) (Fisher *et al.*, 1997) with a broad-spectrum UVB–UVA source. As UVB and UVA1 induce MMP13 mRNA to similar extents (Figure 1h), it is possible that the UVB-induced collagen I hydrolytic activity is also mediated via MMP3 and MMP9. We did not measure MMP3 protein, but UVB resulted in a very large increase in its mRNA that was not seen with UVA1. However, MMP3 protein (stromelysin-1) has been shown to increase after exposure to a UVB–UVA source (Fisher *et al.*, 1997).

UVA1 was more effective than UVB for the degradation of DQ collagen type IV in the epidermis (10 hours). This is primarily a substrate for MMP12, but it is also hydrolyzed by MMP2, MMP7, MMP9, and MMP25 (Yan and Blomme, 2003). As MMP2, MMP7, MMP9, and MMP25 mRNAs were not induced by UVA1, the induction of enzyme activity is more likely to reflect the induction of MMP12. This is also supported by the lack of UVA1 effects on MMP2 and MMP9 protein expression. There is some collagen IV hydrolysis with UVB at 10 and 24 hours that could be due to UVB-induced MMP9 (Figure 3b), although without MMP12-blocking enzymes we cannot rule out the effects of MMP12.

UVB was significantly more effective than UVA1 at inducing epidermal MMP1 activity at 24 hours. In contrast, UVA1 was more potent at induction of epidermal MMP12 protein (24 hours) and its activity (10 hours) against DQ collagen type IV. It was also significantly better at the induction of dermal MMP1 and MMP12 proteins at 24 hours. This difference is likely to be due to greater UVA1 dermal penetration/scattering (Tewari *et al.*, 2011; Tewari *et al.*, 2012). Interestingly, we found no spectral differences for dermal MMP1 and MMP12 activity against their respective collagen substrates, but there was large interpersonal variation. There was some UVB-induced MMP12 protein, predominantly in the epidermis, but to a much lesser extent than with UVA1.

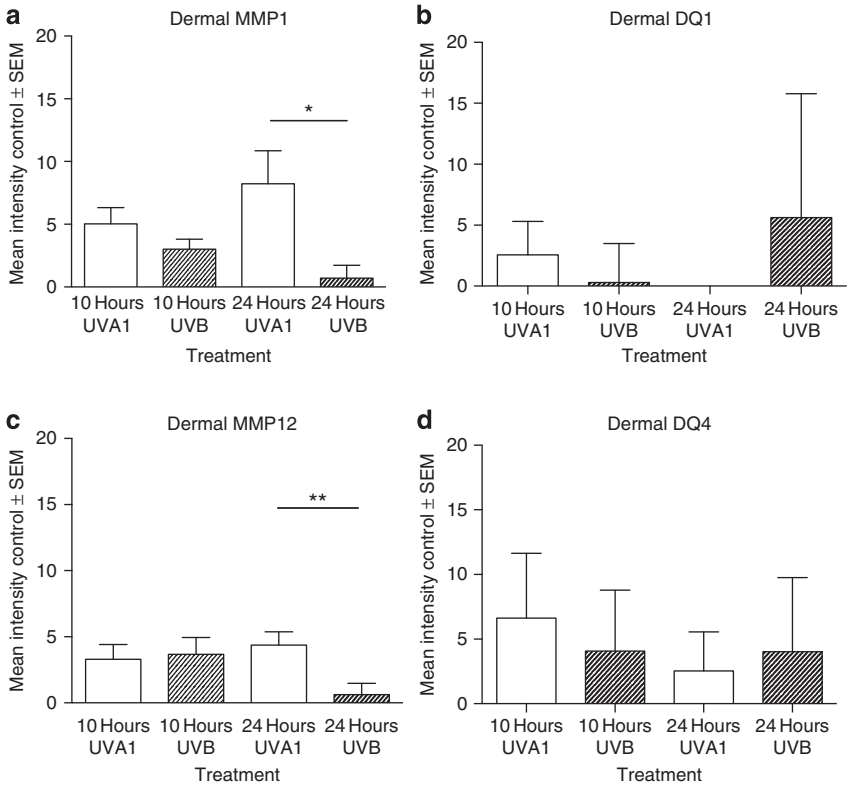

Figure 4. UVA1 induces more dermal metalloproteinase (MMP) protein than UVB. Using immunofluorescence and *in situ* zymography (**a**) there was more UVA1-induced MMP1 than UVB-induced MMP1 at 24 hours, (**b**) there were no spectral differences for the degradation of dermal DQ1, (**c**) there was more UVA1-induced MMP12 than UVB-induced MMP12 at 24 hours, and (**d**) there were no spectral differences for the degradation of dermal DQ4. Scale bar = 50 μm. $*P \leqslant 0.05$, $**P \leqslant 0.01$; $n = 3$.

The small amount (relative to epidermis) of UVA1-induced MMP12 in the dermis, maybe insufficient to degrade elastin at 24 hours because no elastase activity was detected in the dermal region, although this was considerable in the epidermal region. This may be attributed to greater MMP12 protein induction in the epidermis. This is also supported by the *in situ* zymography data for degradation of collagen IV. Essentially, the degradation of two substrates by MMP12 was an epidermal phenomenon in which UVA1 was more effective than UVB.

Dermal extracellular collagens are degraded in photoaging. Quan *et al* (2009) have suggested that epidermal MMP1, MMP3, and MMP9 (where they report the majority is synthesized), diffuse into the dermis to degrade collagen (Quan *et al.*, 2009). However, studies on photoaged skin show more MMPs in the dermis than in the epidermis (Chung *et al.*, 2002; Quan *et al.*, 2013). Overall, this suggests that repeated solar UVR exposure results in an accumulation of dermal MMP, whether by diffusion from the epidermis or by a gradual accumulation of dermally synthesized protein. It is also possible that our 24-hour sampling time was not optimal for MMP12 diffusion from the epidermis into the dermis and the degradation of elastin.

Light microscopy shows that the papillary dermis of photoaged skin contains an accumulation of amorphous disorganized elastin fibrils, which is known as ''solar elastosis'' (Calderone and Fenske, 1995; Yaar and Gilchrest, 2007). In its early stages, there is an accumulation of insoluble disorganized elastin and microfibrillar proteins (fibronectin) (Chen *et al.*, 1986; Lavker and Kligman, 1988), seen clinically as waxy, thickened, and furrowed facial skin. In more advanced solar elastosis, degeneration of dermal elastin results in a mottled appearance that is clinically associated with a loss of skin elasticity. The addition of an elastase to an elastin culture (Braverman and Fonferko, 1982) results in a mottled appearance that is similar to that seen in late solar elastosis. This appearance, termed ''zebra bodies'', can also be induced by repeated erythemal doses of UVA, but not by solar-simulated radiation (Kumakiri *et al.*, 1977).

Our data with gene array, qPCR, and protein activity strongly suggest that UVA1 preferentially induces MMP12. We acknowledge that our protein studies are based on small sample sizes ($n = 3$). However, they generally correlate with the mRNA data, and the protein expression and activity data are mutually consistent. Our elastase activity data (Figure 5) are particularly striking. MMP12 protein has been observed in the upper dermis of patients with solar elastosis and actinic keratosis (Saarialho-Kere *et al.*, 1999; Chung *et al.*, 2002). Chung *et al.* (2002) suggest that MMP12 has a role in elastin remodeling in solar elastosis of the face. We suggest that solar

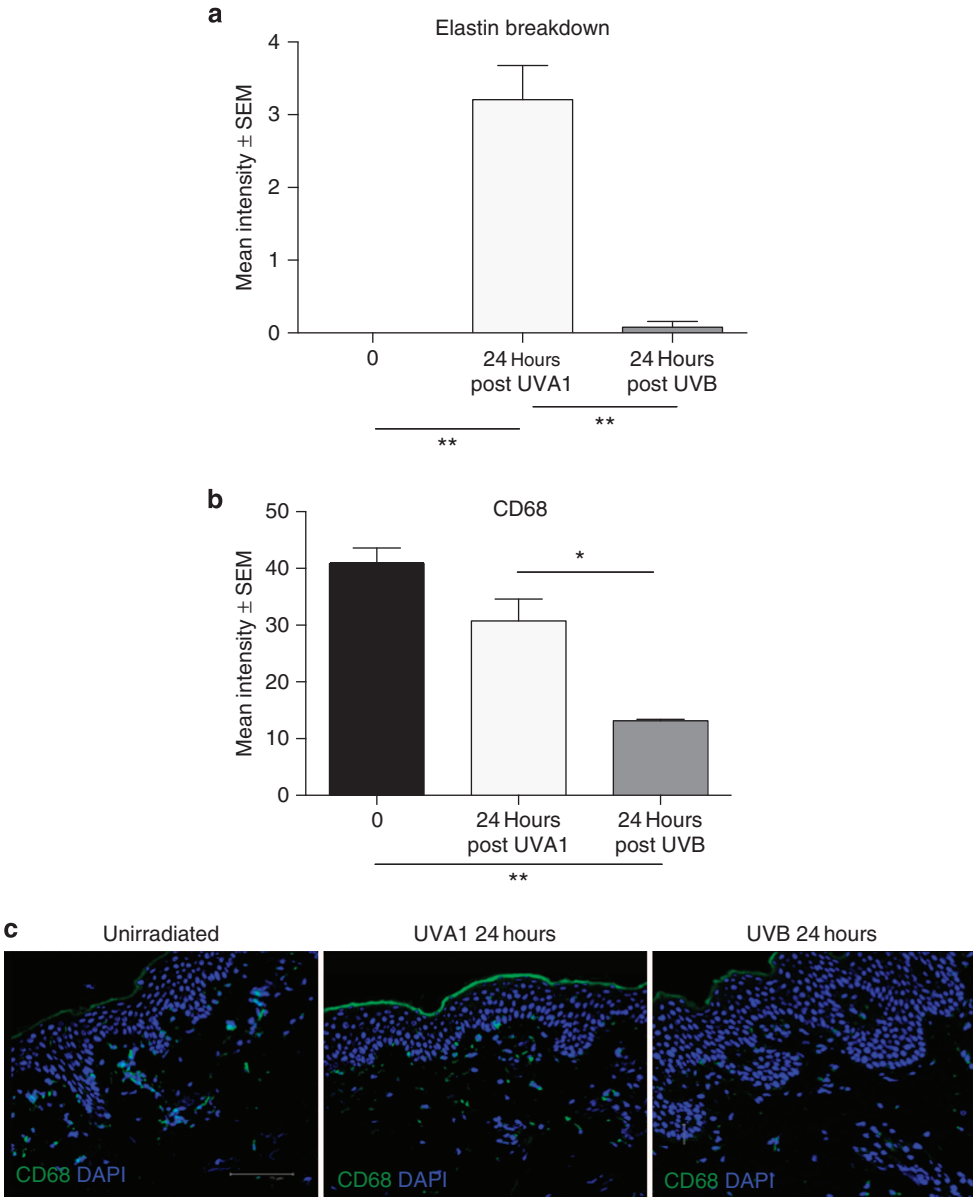

Figure 5. (a) UVA1, but not UVB, initiates DQ elastin degradation. *In situ* zymography showing fluorescence proportional to elastin breakdown at 24 hours after a 1 minimal erythema dose exposure. (**b**) No UVA1 induced depletion of macrophages. Immunofluorescence shows higher numbers of $CD68^+$ macrophages present in the dermis at 24 hours after UVA1 than UVB, (**c**) examples of CD68-positive staining in one individual. $^*P \leqslant 0.05$, $^{**}P \leqslant 0.01$; $n = 3$. Scale bar = 50 μm.

elastosis, and its association with MMP12, may be explained by our studies because MMP12 is the major enzyme for the degradation of elastin (Gronski *et al.*, 1997; Woessner, 1991). However, we cannot exclude other MMPs having a role in the pathogenesis of solar elastosis, because MMP2, MMP7, and MMP9 also have elastolytic activity and may be induced by UVB (e.g., MMP9 as shown in Figure 3b). In addition, MMP12 degrades other substrates apart from type IV collagen including laminin 1, fibronectin, vitronectin, and proteoglycans (Gronski *et al.*, 1997).

We hypothesize that solar UVA1 induces the expression of MMP12, which then degrades elastin, contributing to the loss of skin elasticity seen in late solar elastosis. This may occur, as suggested by Quan *et al* (2009), by diffusion of the MMPs from the epidermis to the upper dermis. The resultant loss of elasticity, termed ''sagging'', has been shown to be a UVA1-dependent process in hairless mice, with an action spectrum peak at 340 nm (Bissett *et al.*, 1987, 1989). This suggests that UVA1 has important implications for photoaging and also possibly photocarcinogenesis (Starcher and Conrad, 1995; Starcher *et al.*, 1996). This is supported by studies that show that people habitually exposed to UVA through glass on one side of the face show more signs of photoaging and skin cancer on the exposed side (Butler and Fosko, 2010; Foley *et al.*, 1986; Mac-Mary *et al.*, 2010; Singer *et al.*, 1994).

Table 1. Volunteer demographics and their just-perceptible MED

Study	Skin type	Sex	Age	MED UVA1 (J cm^{-2})	MED UVB (mJ cm^{-2})
UVA1 time course	I	M	27	48.8	30.0
	II	M	21	48.8	30.0
	I	F	21	61.8	37.0
	I	F	21	61.8	23.0
Mean ± SD		M + F	**22.5 ± 2.6**	**55.3 ± 6.5**	**30.0 ± 4.9**
UVA1 and UVB comparision	I	M	20	61.1	19.0
	I	M	21	61.1	23.0
	II	F	23	61.1	30.0
	I	F	22	61.1	23.0
	II	F	28	76.2	30.0
Mean ± SD		M + F	**22.8 ± 3.1**	**64.1 ± 6.8**	**25.0 ± 4.8**
Protein validation	II	F	24	61.1	37.0
	II	F	27	61.1	37.0
	I	F	22	48.8	30
Mean ± SD			**24.3 ± 2.5**	**57.0 ± 7.1**	**34.7 ± 4.0**
Combined mean ± SD	7I, 5II	4M, 8F	**23.0 ± 2.8**	**59.4 ± 7.7**	**29.1 ± 6.1**

Abbreviations: F, female; M, male; MED, minimal erythema dose.

A reduction of photoaging in an intense solar environment has been observed with the long-term discretionary use of a sunscreen (SPF 15+)(Hughes *et al.*, 2013). Its formulation is not photostable, and would have lost most of its UVA1 protection within 1 hour (Dr B Herzog, personal communication). Our MMP12 data support the inclusion of good photostable UVA1 protection in sunscreens to enhance their ability to inhibit photoaging. Furthermore, our data suggest that the inhibition of MMP12 may be a future strategy for protecting against photoaging.

The absorption of UVR by chromophores mediates all photobiological reactions. UVB absorbed by DNA results in CPD formation, the action spectrum of which peaks at 300 nm in human skin *in vivo* (Young *et al.*, 1998). There is evidence that DNA is a chromophore for MMP1 via CPD formation (Dong *et al.*, 2008). Our recent findings (Tewari *et al.*, 2012; Tewari *et al.*, 2011) showed considerable attenuation of UVB-induced CPDs with skin (epidermis and dermis) depth, which was not the case with UVA1. Thus, we would expect to see marked attenuation of UVB-induced MMP1 with skin depth, as shown in Figures 2g and 4a, if DNA were the putative chromophore. In contrast, epidermal depth has no effect on the distribution of MMP12 induced by UVB or UVA1 (Figure 2i and j). This suggests that they have different chromophores. We therefore propose that UVA1 induces MMP12 via a non-DNA chromophore that generates reactive oxygen species (Scharffetter-Kochanek *et al.*, 1993; Wlaschek *et al.*, 1995). This is supported by studies that showed that topical reactive oxygen species scavengers reduced MMP12 mRNA in human skin (Chung *et al.*, 2002). UVA1 did not induce MMP3 or MMP10 when assessed by qPCR. Our erythemal dose of UVA1 (50 J cm^{-2}) does induce CPDs, although at one-fourth to one-third times lower levels than after an erythemally equivalent exposure to UVB (Tewari, Sarkany *et al.*, 2011). This suggests that these enzymes may not only have a CPD threshold but may also have non-DNA chromophores that are required for their induction.

MATERIALS AND METHODS

Volunteers

The studies were approved by the St Thomas' Hospital, London, UK Ethics Committee (Ref: 09/H0802/98) in accordance with the declaration of Helsinki. The details of the 12 healthy skin type I/II volunteers are shown in Table 1. Participants gave written informed consent before taking part in the study.

Irradiation

UVR sources, dosimetry, and irradiation protocol. Emission spectra and irradiances of the UVA1 and UVB sources, and assessment of MED are previously described (Tewari *et al.*, 2011). Table 1 shows that the mean MEDs for 12 volunteers were 29.2 ± 5.8 mJ cm^{-2} (UVB) and 58.6 ± 7.9 J cm^{-2} (UVA1).

Experimental protocol. (i) ''UVA1 time course'': four skin type I/II volunteers were irradiated over a 1-cm^2 area on previously unexposed buttock skin with 50 J cm^{-2} UVA1 and 4-mm punch biopsies were taken under local anesthesia 6 and 24 hours later. This was ~1MED (minimal erythema dose) and was based on our previous work (Tewari *et al.*, 2011). (ii) ''UVA1 and UVB comparison'': five skin type I/II participants were exposed to 50 J cm^{-2} UVA1 and 30 mJ cm^{-2} (~1MED) UVB and biopsies were taken at 6 and at 24 hours. (iii) ''Protein validation'':

a further three skin type I/II individuals were recruited, had UVR sensitivity tested as above, and received UVB and UVA1 irradiations equivalent to ~1MED (30 mJ cm^{-2} UVB and 50 J cm^{-2} UVA1). Biopsies were taken at 10 and 24 hours along with an nonirradiated control biopsy.

Microarray. A single color hybridization on Agilent Whole Human Genome Oligo Microarrays (Agilent Technologies, Waldbronn, Germany) (4 × 44 K) and bioinformatics was performed (Milteyni Biotech, Bergisch Gladbach, Germany). Briefly, biopsies were snap-frozen in liquid nitrogen and RNA was extracted (Trizol, Sigma, St Louis, MO) and quality checked (Agilent 2100 bioanalyzer platform; Agilent Technologies). cRNA was produced, Cy3 labeled, and hybridized overnight (~17 hours, 65 °C) to Agilent Whole Genome Oligo Microarray chips (4 × 44 K) using the Agilent Gene Expression Hybridzation Kit (Agilent Technologies). Fluorescence signals were detected using Agilent's Microarray Scanner system, Agilent Feature Extraction Software was used to process intensities. Raw intensity data were extracted from Feature Extraction output files using Rosetta Resolver software (Rosetta, Inpharmatics, Kirkland, WA). Background-corrected intensity values were normalized between arrays using quantile normalization (Bolstad *et al*., 2003). Log_2-transformed normalized intensity values were used for subsequent statistical analysis. Quality controls include comparison of intensity profiles and a global correlation analysis. The microarray data is deposited at NCBI GEO with accession number GSE45493.

Quantitative real-time PCR

Total RNA was converted to cDNA (Applied Biosystems, Paisley, UK) and quality controlled using reverse transcription PCR with β-actin as the housekeeping gene. cDNA was visualized as characteristic bands on a 3% agarose gel under UVR. qPCR was performed using Taqman Gene Expression Assays (Applied Biosystems) according to the manufacturer's protocol with the following TaqMan DNA probes (Applied Biosystems), FAM (6-carboxyfluorescein) labeled, and GAPDH (Applied Biosystems) housekeeping gene, VIC labeled. Probes used (gene name and reference no): MMP1 Hs00899658_m1, MMP3 Hs00968305_m1, MMP9 Hs00234579_m1, MMP10 Hs00233987_m1, MMP12 Hs00899668_m1. Fold change was calculated using the ΔΔCT method.

Protein validation

Biopsies were placed in OCT (VWR Chemicals, Leuven, Belgium), embedded in isopentane (VWR Chemicals, Fontenay-sous-Bois, France), and once placed in cryovials were then frozen in liquid nitrogen. 5–7 μm sections were mounted on Superfrost plus slides and stored at −80 °C. Sections were placed at room temperature (10 minutes), fixed in cold acetone (10 minutes), then rinsed in phosphate-buffered saline (PBS) for 5 minutes. (i) Immunofluorescence: sections were incubated with blocking buffer for 20 minutes (10% goat serum (DAKO, Cambridge, UK) 0.1% BSA, 0.1% Tween-20 in PBS), washed in PBS, incubated for 90 minutes with MMP1 (1:100), MMP12 (1:400) (recognizes the proactive and active forms of MMP12), rabbit antihuman antibodies (Abcam, UK), CD68 (1:100) mouse antihuman antibody (Abcam, Cambridge, UK) washed in PBS, and incubated with either Alexa Fluor goat antirabbit 555 or goat antimouse 555 (Invitrogen, Paisley, UK) at 1:200 for 30 minutes, counterstained with prolong gold antifade with DAPI (Molecular Probes, Paisley, UK), coverslipped, and stored away from light. Imaging was performed with a Zeiss Axiophot microscope (Harpenden, UK) and Nikon DS-U2 camera (Kingston upon Thames, UK). Images were captured in 2560 × 1920 format, gain × 1.00, 4 second exposure. (ii) *in situ* zymography: slides were incubated with 60 μl substrate (low-gelling agarose (Sigma, Dorset, UK) (1 g dissolved in 500 ml PBS)), with four drops of 4′,6-diamidino-2-phenylindole with antigold fade (Invitrogen) and DQ-collagen 1, DQ-collagen 4, or DQ elastin (all from Molecular Probes) at 1:10 for ~18 hours in the dark. Fluorescent imaging for fluorescein (green) and 4′,6-diamidino-2-phenylindole (blue) was performed. Images were obtained as above and captured in 2560 × 1920 format, gain × 1.00, 1 second exposure. Analysis was performed using NIS elements BRv2 software package as previously described (Tewari *et al*., 2011) after removing background control intensity. For the spread of MMP1 and MMP12 across the epidermis, thresholding was set to capture red intensity, and upper epidermis and lower epidermis were visually gated to give mean intensity values corresponding to MMP amount.

Data analysis

For microarray data, bioinformatical analysis was based on normalized Log_2 intensities using R/Bioconductor and software packages therein (http://www.R-project.org; http://www.bioconductor.org). Analysis of variance with repeated measurements followed by Tukey *post hoc* tests was used to test for expression differences among the groups. The UVA1 time course was as follows: UVA1 6 hours versus UVA1 24 hours (50 J cm^{-2} doses), UVA1 versus UVB at 6 hours, and UVA1 versus UVB at 24 hours. All *P*-values were adjusted for multiple testing (Benjamini and Hochberg, 1995). Differentially expressed genes were considered if both the analysis of variance and Tukey *post hoc* test *P*-values were $\leqslant 0.05$ (adjusted *P*-value or false discovery rate $\leqslant 0.3$) and the expression difference was at least twofold.

Functional analysis of candidate genes was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) v6.7 (Dennis *et al*., 2003) to identify biological clusters and GeneGo Metacore v7 to identify key pathways. Briefly, gene ratio lists for pooled individuals (fold change $\geqslant 2$, $P \leqslant 0.05$) from the 6- and 24-hour biopsies were uploaded to the software program, which generated pathway maps and *P*-values associating the statistical likelihood of a sequence of genes with a particular pathway. All graphs were generated using the Graphpad Prism v4 statistics package.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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UVA1 is skin deep: molecular and clinical implications†

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Long wavelength UVA1 (340–400 nm) is the main component of terrestrial UVR and is increasingly used in skin phototherapy. Its damage to critical biomolecules such as DNA has been widely attributed to its ability to generate reactive oxygen species (ROS) *via* other chromophores. However recent studies *in vitro* and *in vivo* have shown that UVA1 has a specific ability to generate cyclobutane pyrimidine dimers (CPD), especially thymine dimers (T<>T), and that this is probably due to direct absorption of UVR. The CPD has been implicated in many aspects of skin cancer. Measuring UVB-induced CPD in the epidermis and dermis *in vivo* shows that, as expected, the skin attenuates UVB. In contrast, our data show that this is not the case with UVA1: in fact there is more damage with increased skin depth. This suggests that the basal layer, which contains keratinocyte stem cells and melanocytes, is more vulnerable to the carcinogenic effects of UVA1 than would be predicted by mouse models. These data support the continuing trend for better UVA1 protection by sunscreens.

Erythema as a biological measure of exposure to solar UVR

Ultraviolet radiation (UVR) is electromagnetic radiation spanning 100–400 nm and is officially divided into UVC (100–280 nm), UVB (280–315 nm) and UVA (315–400 nm), with UVA being sub-classified into UVA2 (315–340 nm) and UVA1 (340–400 nm). However, a UVB–UVA cutoff at 320 nm is widely used in photodermatology. Since stratospheric ozone absorbs all UVR below ~295 nm, terrestrial sunlight contains only UVB (5–10% of total UVR) and UVA (90–95% of total UVR), the latter being primarily UVA1 (~75%). The precise ratio of UVB to UVA varies with the solar zenith angle that is determined by latitude, season and time of day. This is demonstrated by Fig. 1a, which shows the monthly variation of UVA and UVB irradiance at Chilton, UK (51.6°N), in which a radiative transfer model (SMARTS)[1] generated the data taking total ozone into account.[2] Similarly, Fig. 1b shows the daily variation of

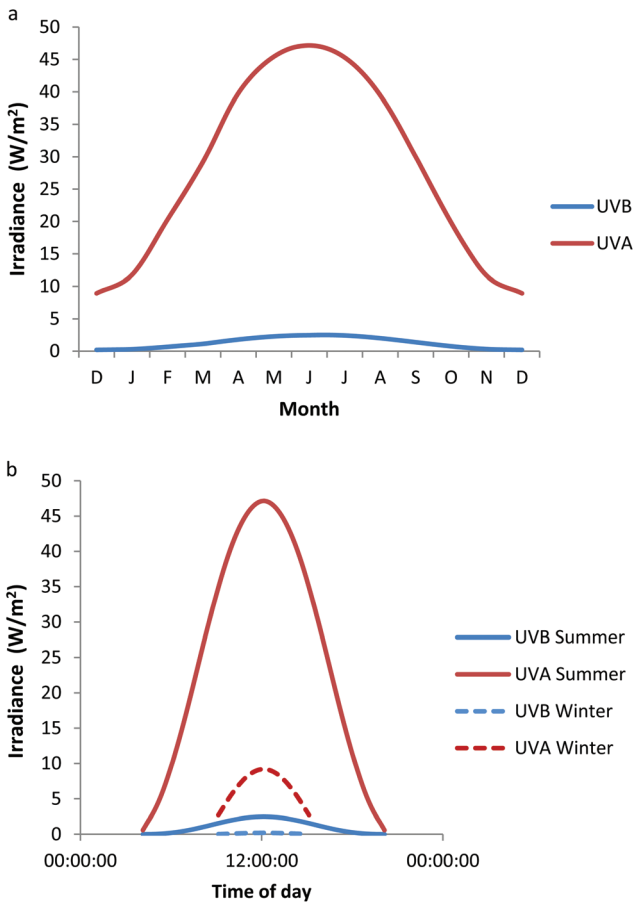

Fig. 1 Variation of UVB and UVA irradiance at Chilton, UK (a) monthly at noon on the 21st day of each month (b) daily at summer and winter solstices. These data have been modeled taking total ozone into account.[2]

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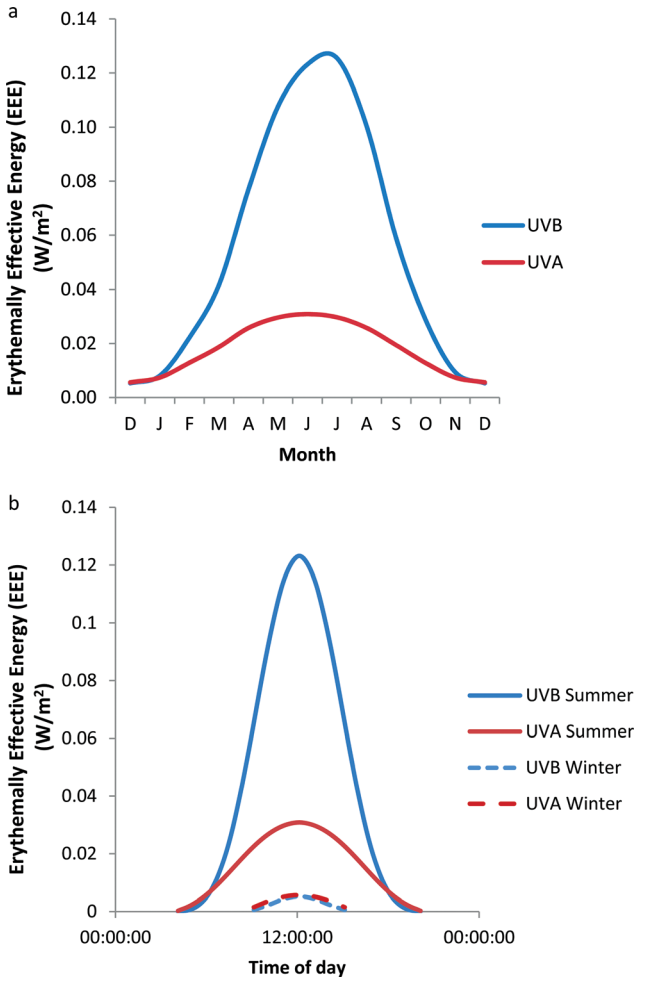

Fig. 2 Variation of UVB and UVA EEE at Chilton, UK (a) monthly at noon on the 21st day of each month (b) daily at summer and winter solstices. These data are based on the data in Fig. 1.

UVA and UVB taken in mid-summer (21st June) and mid-winter (21st December) at the same location.

Erythema is widely used as a clinical measure of UVR exposure. Thus, the data in Fig. 1a and 1b have been weighted with the CIE erythema action spectrum[3] to produce erythemally effective energy (EEE) as shown in Fig. 2a and 2b respectively. Fig. 2a shows that in mid-summer, UVA at temperate latitudes contributes about 25% of EEE of solar UVR. However, this decreases either side of this period such that the ratio is approximately 50 : 50 from December through to January. Fig. 2b shows that UVA contributes to a maximum of about 25% of EEE at noon in mid-summer, but that in mid-winter the relative contributions of UVB and UVA to erythema are independent of time of day, *i.e.* erythemal exposure is achieved equally by UVB and UVA. In this context, it must be remembered that considerable molecular and cellular damage can be done in human skin *in vivo* by single and repeated sub-erythemal exposure,[4] irrespective of spectrum. Furthermore, suppression of the skin's immune function can occur with about 25% of the minimal erythema dose (MED).[5]

The standard erythema dose (SED)[6] is increasingly used as a measure of human exposure, especially in epidemiological studies. Unlike the MED, the SED is independent of individual UVR sensitivity. During mid-summer in Chilton at midday it would take approximately 9.5 minutes to receive a full UVR spectrum SED (unpublished data) which is approximately 1/3 of a minimal erythema dose (MED) in a skin type I/II person.[7] It would take approximately 4 times longer to get an SED from the UVA component alone. These data show considerable potential exposure to UVA whether determined by physical or erythemal exposure parameters.

UVR and skin cancer

There has been a dramatic increase in incidence of skin cancers in Europe and the USA, especially malignant melanoma (MM), in the past 25 years.[8,9] This has increased the urgency of identifying and protecting against the causative factors of which UVR has emerged as the most important. This conclusion has resulted from classic epidemiology and more recently, by molecular epidemiology that identifies UVR-specific mutations in skin cancers. The focus has been largely on UVB because of a combination of action spectrum data from a mouse model of squamous cell carcinoma (SCC)[10,11] and the well-established finding that UVB readily induces the production of DNA photoproducts (see section below) associated with skin cancer.[12–14] However, a combination of factors has arisen in recent years that implies that UVA plays a more significant role than was previously thought. First, epidemiological data have shown that MM shows a better latitude correlation with UVA than with UVB[15,16] and that the use of artificial tanning devices (which mainly emit UVA) increases the risk of skin cancer, especially MM.[17] UVA artificial tanning devices are widely used,[18] especially by the young, and use under the age of 35 confers the greatest risk.[19] Second, it has become increasingly clear that UVA1 is not photochemically or biologically inactive, and that UVA1 is a potent inducer of reactive oxygen species (ROS),[20] which are thought to play a role in skin cancer.[21,22] The question of whether UVA1 is a significant carcinogen has become more important as exposure of the human population to UVA has significantly increased. Until relatively recently, sunscreens were primarily UVB absorbing products that enabled prolonged sunbathing without much protection against UVA, paradoxically increasing exposure to UVA in sunbathers.[23,24] This can occur to the extent that erythema from repeated sub-erythemal exposure can be caused by UVA.[25] Finally UVA1 phototherapy has been developed in the last 20 years and is used to treat a variety of dermatological diseases[26] with comparable efficacy to other types of phototherapy (TLO1 or narrowband UVB) for some conditions. Although the action spectrum for induction of SCC in mice is predominantly in the UVB waveband, the action spectrum for induction of MM in mammalian skin is still unknown. Mechanistically, the proven mutagenicity and carcinogenicity of UVB has led to the dogma that UVB is the only significant cause of skin cancer.

The rest of this review will focus on what is currently known about UVA1 photodamage of DNA, the significance of this damage in terms of mutagenicity and carcinogenicity, and the clinical and public health implications of these new findings.

UVR-induced DNA damage

DNA is susceptible to modification by UVR which may result in mutation if not successfully repaired. There are many different types of DNA photolesions, which show wavelength dependence in their formation. Some are formed by the direct absorption of UVR and others are formed indirectly as described below.

(i) Di-pyrimidine photolesions

UVR is absorbed by skin chromophores[27] and DNA pyrimidine bases are primarily chromophores for UVB (thymine, cytosine and the minor 5 methylcytosine),[28] but also weakly absorb UVA. The direct excitation of these nucleobases by UVB in an oxygen independent manner induces two main classes of photolesions in the DNA of skin fibroblasts and keratinocytes.[29] These are cyclobutane pyrimidine dimers (CPD) and 6-4 pyrimidine pyrimidone adducts (6-4PP) (which make up 65% and 35% of the UVB induced DNA lesions respectively.[30,31] The CPD arises by linkage of two adjacent pyrimidine bases of which the T<>T type is the most common.[32]

Until recently the presence of CPD-induced "fingerprint" mutations (*e.g.* C→T) in skin cancers was taken as *a priori* evidence for a causal role for UVB, even though it has been known for some time that UVA, including UVA1, induces CPD in human skin *in vivo*.[33,34] More recent studies have confirmed that UVA1 induces CPD *in vitro*, *ex vivo* and *in vivo*[32,35] but not 6-4PP,[32,35] these being predominantly (but not exclusively) T<>T at frequencies comparable to or exceeding that of oxidative damage to DNA (see below) but at several orders of magnitude lower efficiency than UVB. The dogma that C→T mutations are only caused by UVB has been recently challenged.[36]

Distribution of UVB and UVA1-induced CPD in human skin *in vivo*. Fig. 3 shows the distribution of CPD and 6-4PP in the epidermis and dermis when biopsies were taken immediately after erythemally equivalent does of UVA1 (Fig. 3a) and monochromatic UVB at 300 nm (Fig. 3b); the latter wavelength was chosen because it approximately corresponds to the peak for the action spectra for both erythema and CPD.[33] Fig. 3b shows that both types of UVB-induced-damage are attenuated with increasing skin depth, as would be expected because of the presence of UVB absorbing chromophores in the skin. In contrast, Fig. 3a shows a significant increase in CPD with skin depth with UVA1 and no evidence for 6-4PP. These figures show that UVB and UVA1 can cause significant DNA damage to dermal cells; such damage has been largely neglected in the past, especially with UVB. Assessments of the epidermis showed that UVA1 induces at least twice as much DNA damage

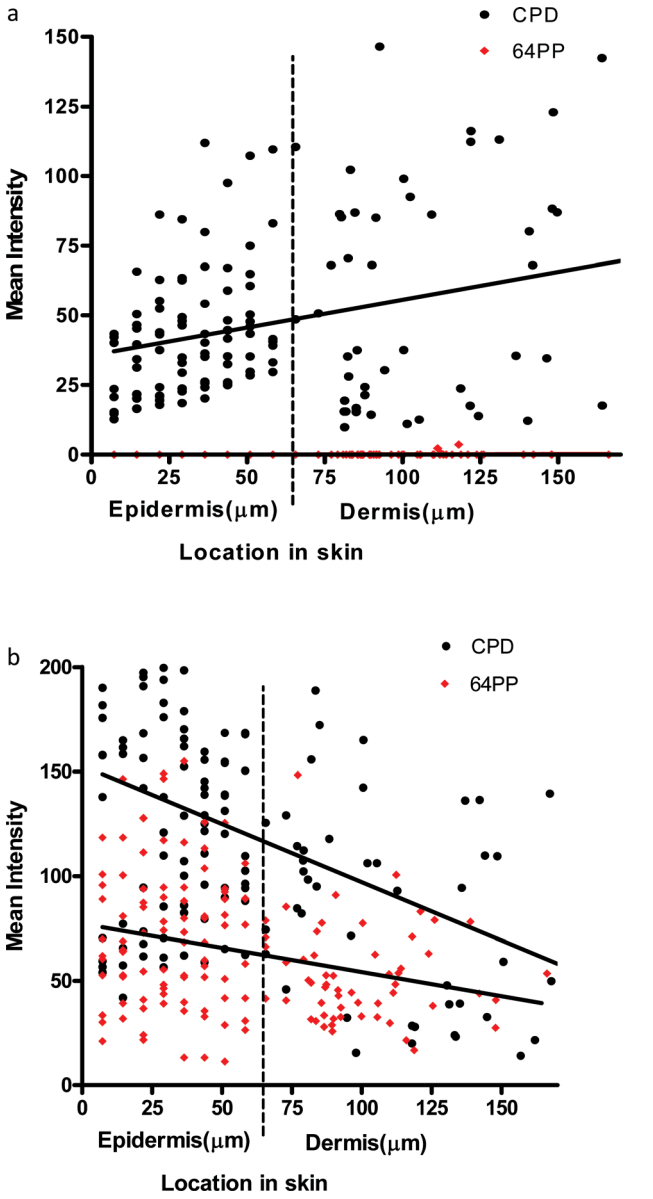

Fig. 3 (a) Linear regression for UVA1-induced CPD (p = 0.0006 for slope) and 6-4PP (p = 0.56 for slope) in the epidermis and dermis *in vivo* immediately after a 3MED exposure. More CPD appear to be induced as depth increases through the epidermis and dermis. Note the lack of UVA1 induced 6-4PP. (b) Linear regression for UVB (300 nm)-induced CPD (p = <0.0001 for slope) and 6-4PP (p = 0.0006 for slope) in the epidermis and dermis *in vivo* immediately after a 3MED exposure.

in the basal layer than the upper epidermis.[35] Thus, the extent of CPD damage varies with spectrum and epidermal location.

UVA1 is less well absorbed by the upper layers of the epidermis than UVB and hence can penetrate deeper into tissue, but the reasons for the "reverse attenuation" with UVA1 are not known. One possibility is that UVA1 photons are preferentially scattered by the epidermis and dermis, in a forward or backward direction, rather than absorbed by chromophores.[37,38] Back scattering results in remittance (a type of "reflection"), which provides additional opportunity for chromophore

absorption during the return pathway. Dermal remittance increases between 300 and 400 nm.[37] Thus, it is possible that the higher number of UVA1-induced CPD seen in the basal layer is due to dermal back scatter (*e.g.*, from collagen), as well as epidermal forward scatter.[39]

Although we have only measured CPD and 6-4PP, these can be used as a surrogate for UVR penetration into skin and can inform about the possibility of generating other types of DNA and bio-molecule damage. Overall, our data demonstrate that the basal layer, which contains melanocytes and proliferating keratinocytes, is especially sensitive to UVA1 induced DNA damage, and they challenge the paradigm that UVB is the only important spectral region for skin cancer. This is supported by studies on engineered human skin show that, in contrast to UVB, UVA-induced mutations were mainly located in the basal layer.[40]

The photochemical mechanisms of UVA1 CPD induction. The photochemical mechanisms, by which UVA1 selectively induces CPD, especially T<>T, and not 6-4PP (see Fig. 3a) *in vivo*, remains unknown. UVA activation of some photosensitizers such as fluoroquinolones[41] and carprofen[42] can induce CPD *in vitro*, suggesting that unidentified endogenous photosensitizers in human skin *in vivo* may have a role in the induction of CPD by UVA1, possibly *via* photosensitizer mediated triplet energy transfer.[43,44] More recently a direct UVR absorption photochemical reaction[45,46] has been proposed from *in vitro* studies. For example, double-stranded base sequences (*e.g.*, dA.dT) increase UVA absorption compared with the same monomeric molecules.[46]

(ii) Oxidative damage

DNA damage also occurs indirectly through the formation of ROS.[44,47] These are generated by the absorption of UVA by non-DNA endogenous chromophores (photosensitizers) such as porphyrins, flavins and NADH/NADPH[44,48] which can be excited to a triplet state. Damage can occur *via* a type 1 photosensitization reaction which can cause DNA strand breaks by the generation of superoxide anions, hydrogen peroxide and hydroxyl radicals, the latter being very reactive with all DNA bases.[47] There are also type 2 photosensitization reactions where energy is transferred to molecular oxygen giving rise to singlet O_2 ($^1O_2$). This species specifically reacts with guanine residues[49] producing 8-oxo-7,8-dihydro-2′deoxyguanosine (8oxodG) and is thought to be important in UVA induced cell death.[50] Few studies have reported the detection of UVR-induced oxidative damage to DNA *in vivo*, but this has been reported in mice[51] and in human skin.[22,52] 8oxodG has also been reported in urine after the exposure of human skin to UVA.[53]

When UVB (300 nm) and UVA1 doses are given to produce comparable levels of erythema in human skin (1MED ~ 30 mJ cm^{-2} and ~50 J cm^{-2} respectively), UVA1 induces 3–4 fold fewer T<>T than UVB implying other non-DNA chromophores have an important role in UVA1 erythema, and it is possible that this is mediated *via* oxidative damage to DNA. Unlike UVB erythema, UVA erythema is oxygen dependent,[54] implying a role for ROS in UVA erythema. Different mechanisms for UVB and UVA1 erythema are supported by a lack of correlation between UVB and UVA1 induced MED on the same individuals (Fig. 4). A detailed laser action spectrum for human erythema showed a peak at 300 nm but also identified a minor UVA1 peak (at approximately 360 nm)[55] which also suggests that more than one chromophore is important (Fig. 5).

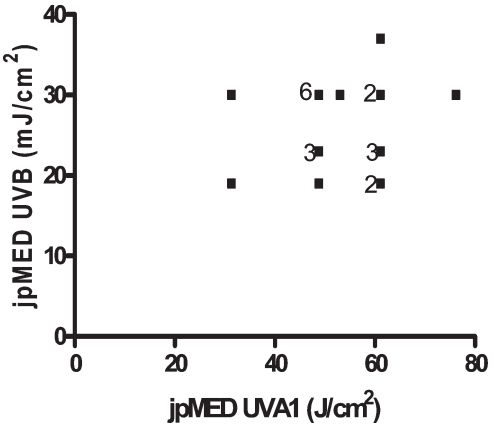

Fig. 4 Lack of relationship between a given individual's UVB and UVA1 MED (n = 22 with some data points overlapping). jp = just perceptible.

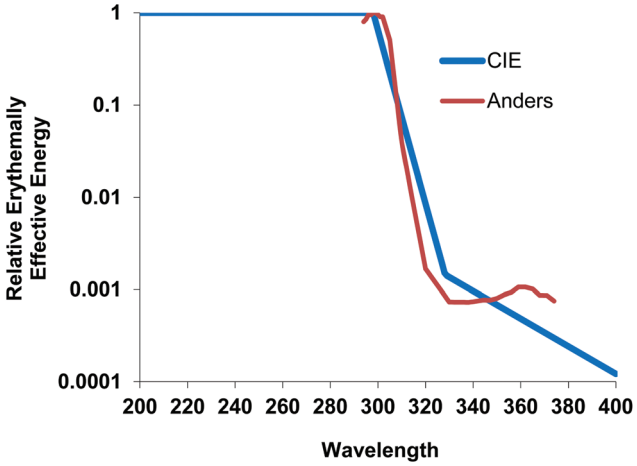

Fig. 5 Erythema action spectrum[55] showing small peak at approximately 360 nm (raw data obtained from the late Dr Anders) normalized at 300 nm with the CIE action spectrum.[3]

Consequences of UVR induced DNA damage in human skin

(i) Non-genetic

The action spectra for CPD and erythema (inflammation) are similar (especially between 280–340 nm) suggesting that DNA is a chromophore for erythema[33] which is also supported in studies in the marsupial *Monodelphis domestica* in which the MED was reduced by a factor of approximately four after enhanced CPD repair induced by UVA activation of

endogenous photolyase which is not present in placental mammals.[56] In other words the CPD triggers inflammation of the skin. Inflammation plays a role in many types of cancer[57,58] by fostering tumour progression, and UVR-induced erythema is a risk factor for MM and possibly BCC.[59] Persistent erythema is also found in xeroderma pigmentosum patients who are unable to repair CPD and who are also 2000–10 000 times more likely to present with melanomas and non-melanoma type skin cancers.[12,60]

Apart from erythema, the CPD has been shown to trigger pro-inflammatory cytokine release and immunosuppression that are thought to play a role in skin cancer.[61,62] We have recently shown that 3 SED, irrespective of spectrum (including UVA1), is predictive of the suppression of the sensitization phase of the contact hypersensitivity (CHS) response, which is regarded as a model for the immunological events in non-melanoma skin cancer (manuscript in preparation). In conclusion, there is considerable evidence that DNA damage, especially the CPD, causes inflammation/immunosuppression that may play a role in skin cancer. Recently published work also suggested that erythema is predictive of photoageing induced by matrix metalloproteinase 1 (MMP1) *via* the CPD.[63]

Oxidative damage and immunosuppression

The CPD and urocanic acid have been identified as possible chromophores for UVR-induced immunosuppression.[64,65] However several studies in the mouse[66,67] have shown that antioxidants can inhibit UVR-induced immunosuppression, which suggests other possibly UVA absorbing chromophores. In addition, antioxidants have been shown to inhibit mouse photocarcinogenesis.[68,69] However, there is no direct evidence that oxidative damage to DNA is a trigger for UVA-induced immunosuppression.

(ii) Genetic

Di-pyrimidine lesions. Di-pyrimidine lesions containing a cytosine are thought to lead to a C→T transition (C:G to A:T transition) which are commonly found in UVR induced skin cancer (SCC, basal cell carcinoma (BCC)),[14] and the majority of mutations in a melanoma cell line[70] but rarely in non-UVR-induced internal cancers. Thus cytosine-containing photoproducts are thought to be more mutagenic. C→T transitions can be induced by both CPD and 6-4PP. However in mammalian cells, 6-4PP are repaired quickly[71,72] whilst the CPD is believed to be the major mutagenic lesion owing to its high level of induction, slow repair by nucleotide excision repair (NER) and efficient replication bypass.[73] Transgenic mouse studies have shown that the CPD is much more important for non-melanoma skin cancer than the 6-4PP.[74]

Faithful NER of CPD is crucial for the prevention of skin cancer. One study on *ex vivo* skin suggested poorer repair of UVA1-induced CPD compared with those induced by UVB[32] but our data did not support this when the epidermis was assessed in total.[35] However, a subsequent analysis of repair in the basal layer alone suggests that repair of UVA induced CPD is less efficient than that with UVB (unpublished). UVA can induce a G2/S block in melanocytes and melanoma cells suggesting that the DNA damage induced by UVA can have potent effects as they may not be easily repaired at the basal epidermis.[75] Recent work[76,77] suggests that UVB initiates melanoma and that both UVB and UVA are involved in the progression of the disease.

ROS-induced lesions. 8oxodG can induce G→T transversions which are potentially mutagenic,[78,79] but because ROS-mediated base damage is low compared to direct damage and because this type of damage is rapidly repaired[80] the importance of these lesions in skin carcinogenesis is less clear.[32,43,81]

Oxidative damage to DNA results in what have been termed "UVA fingerprint mutations" *via* G:C→A:T transversions which have been detected in human SCC and actinic keratosis, especially in the basal layer.[22] These were found to be more prominent than C→T, transitions[21] but p53 gene mutations do not contain UVA specific T and G transversions implying that although UVA causes oxidative DNA damage, such lesions may not contribute to the generation of p53 mutations.[82] Also, the UVA mutation spectrum in mammalian cells does not exhibit a predominance of G:C→T:A transversions[83] which, combined with the lack of increase in mutation rate in cells deficient in repair of 8oxodG,[84] suggests that other lesions are involved in UVA mutagenesis. New work in mice[85] examined UVB and UVA induced DNA damage and their contribution to melanoma. UVB induced melanoma was independent of melanin, but UVA-induced melanoma required melanin. UVA, but not UVB, induced oxidative DNA damage in melanocytes of pigmented mice. These data support other work that suggests that melanocytes are particularly sensitive to UVA.[86]

Clinical implications of UVA1 DNA photodamage in the skin

As discussed earlier, potential exposure to solar UVA1 is substantial, even in temperate UK climates. Further south, on a summer's day in the south of France the maximum ambient daily UVR dose is ~22MED and the maximum UVA dose is ~137 J cm^{-2},[23] of which the majority is UVA1. The accumulation of high UVA1 doses also occurs during the treatment of dermatological skin disorders such as morphea[87,88] and atopic eczema[89] where on average patients receive cumulative doses of approximately 900 J cm^{-2} UVA1 (for example 50 J cm^{-2} 3 times a week for 6–8 weeks) and some conditions such as atopic eczema of the hands or cutaneous T-cell lymphoma receiving single doses of up to 100 J cm^{-2} UVA1.[90] The long-term consequences of such exposures are not known. We have shown that repeated daily exposure to sub-erythemal doses of solar simulated radiation (SSR) results in an accumulation of CPD[4,25] probably because repair of this lesion is relatively slow. To the best of our knowledge, the effects of repeated UVA1 sub-erythemal exposure on CPD has not been studied.

A recent study[91] demonstrated that uncontrolled activation of a major mediator of the Hedgehog pathway signalling can lead to the development of nodular BCC from hair follicle stem cells and superficial BCC-like tumours from the basal cell compartment of the epidermis. Such cells may also thus be vulnerable to UVA1 because of its skin penetration properties.

Increasing recognition of the possible long-term harmful effects of solar UVA exposure and development of better UVA sunscreens is now required by regulatory bodies in Europe and the USA.[23] Recent government regulation in the UK and other countries and prohibits the use of sunbeds for those under 18 years of age.

Conclusions

Our data, and those of others, show that the basal epidermis of human skin is especially vulnerable to UVA1-induced CPD, and ROS-induced mutations and therefore suggests that the carcinogenic potential of this spectral region, which is the major UVR component of sunlight, may be much greater than has been estimated for SCC in the albino mouse, which has a very thin epidermis and unlikely to show much evidence of differential UVA1 induced damage in the epidermis.

The UVA1 sensitive basal layer is the location of proliferating keratinocytes and melanocytes. The action spectrum for MM in mammalian skin is unknown, but there are epidemiological data to suggest a greater role for UVA for MM compared to non-melanoma skin cancer, furthermore recent mouse data suggest that melanocyte DNA is especially sensitive to oxidative damage.[85] Overall, the data support the use of broad-spectrum sunscreen protection.

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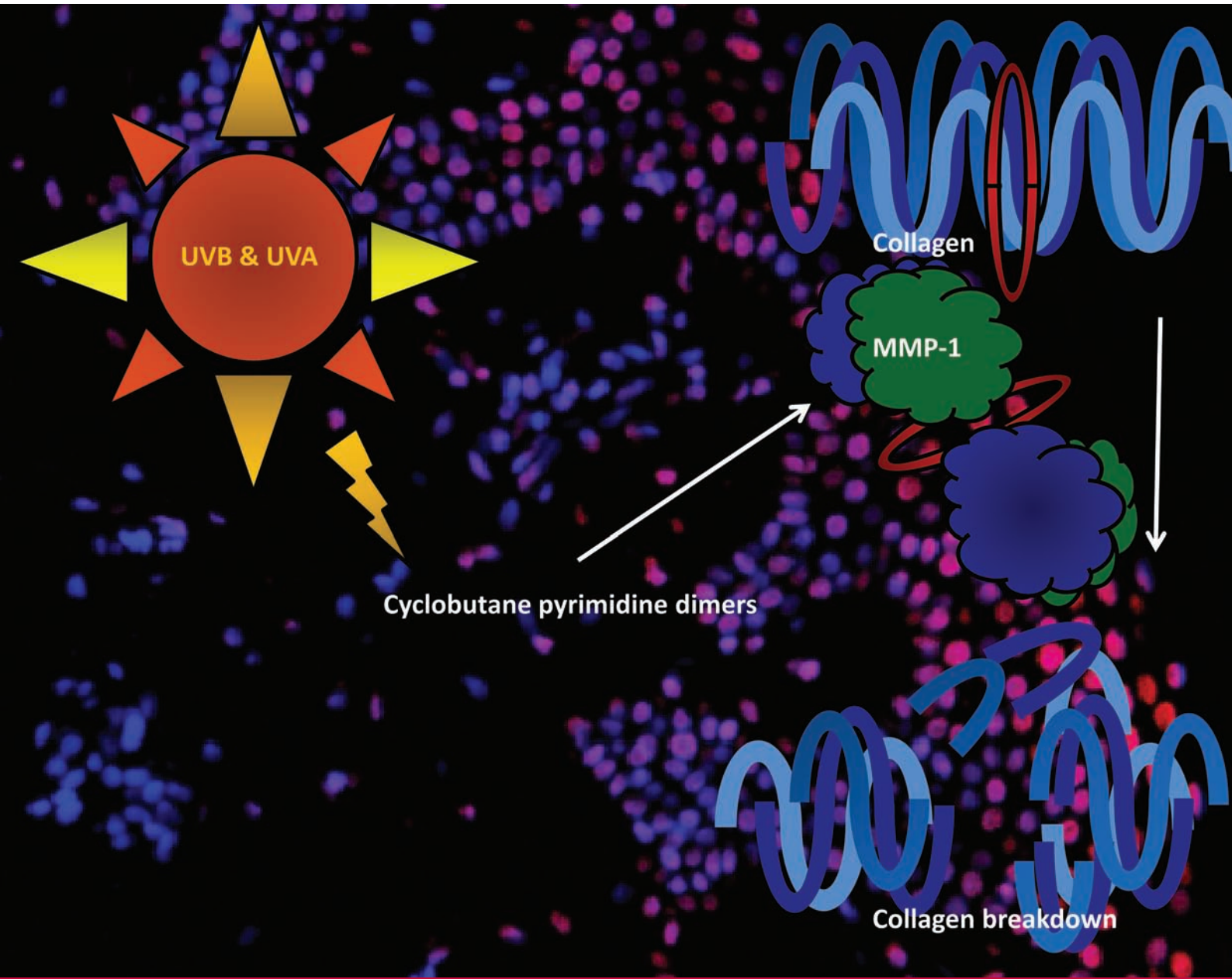

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PAPER

Human erythema and matrix metalloproteinase-1 mRNA induction, *in vivo*, share an action spectrum which suggests common chromophores†

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Matrix metalloproteinase 1 (MMP-1) is widely regarded as a biomarker of photoageing. We tested the hypothesis that MMP-1 mRNA expression and erythema share a common action spectrum by comparing the effects of erythemally equivalent doses of UVB, UVA1 and solar simulated radiation (SSR) on acute MMP-1 mRNA expression in whole human skin *in vivo*. Our results show comparable MMP-1 expression with all three spectra, which supports our hypothesis. The sharing of an action spectrum implies common chromophores, one of which is likely to be DNA. We have previously shown that all spectra that we used readily induce cyclobutane thymine dimers (T<>T) in human epidermis *in vivo* but we lack quantitative data on damage to dermal DNA. This is important because we do not know if dermal MMP-1 induction occurs *via* direct damage to the dermis, or indirectly *via* damage to the epidermis. Our results show that UVB induces about 3 times more T<>T compared with erythemally equivalent doses of UVA1, which is similar to our published epidermal data. This supports previously published work that also implicates an unknown UVA1 chromophore for erythema and MMP-1 induction. However, the distribution of the dermal DNA damage varies considerably with spectrum. In the case of UVB it is primarily in the upper dermis, but with UVA1 it is evenly distributed. Thus, irrespective of chromophores, MMP-1 induction by direct dermal damage by both spectra is possible. The practical conclusions of our data are that the small (<5%) UVB content of solar UVR is likely to be the main cause of photoageing, at least in terms of MMP-1 expression. Furthermore, prevention of erythema by sunscreen use is likely to result in reduced MMP-1 expression.

Introduction

Photoageing and skin cancer are the normal long term consequences of exposure to solar ultraviolet radiation (UVR: ~295–400 nm), especially in sun sensitive skin types I and II. Skin cancer is a major health problem and has been subject to extensive epidemiological and basic research. There has been relatively limited research into photoageing, even though this is often associated with skin cancer, and mouse studies have shown that neutrophil elastase deficient hairless mice are resistant to photoageing and UVR-induced squamous cell carcinoma (SCC), suggesting a mechanistic link.[1]

The vast majority (>95%) of solar UVR is UVA (320–400 nm), of which UVA1 (340–400 nm) is the major (~75%) component, but action spectroscopy has shown that the much smaller (<5%) UVB (~295–320 nm) component is responsible for the majority of DNA photodamage (thymine dimers; T<>T) in human skin *in vivo* and sunburn;[2] this has led to the conclusion that DNA is an important chromophore for erythema. It is also widely accepted that UVB is the main cause of skin cancer,[3] though some have advocated an important role for UVA, especially for malignant melanoma.[4,5] UVA1 is increasingly used in high dose phototherapy for skin disorders[6] and is typically the major spectral component of tanning devices.[7]

Most research on the effects of UVR on skin *in vivo* has focused on the epidermis because it is the target tissue for photocarcinogenesis. The dermis is also an important target, especially in the context of inflammation and photoageing.[8] The structural integrity and function of the dermis are dependent on its extracellular matrix (ECM), which is mainly composed of type I and type II collagen.[9] Collagen turnover is mediated by matrix metalloproteinases (MMP)[10] and their inhibitors (TIMP). MMP-1 is highly induced by UVR in resident keratinocytes and fibroblasts, as well as MMP-3 and MMP-9 *in vivo*.[10–12] Photoageing is thought to be caused by UVR-induced hydrolysis of the dermal ECM that is initiated by MMP-1; a blocking antibody to MMP-1 removed 95 ± 4% of collagenolytic activity from UVR-exposed human skin, strongly implicating MMP-1 as a major enzyme responsible for collagen degradation in photoageing.[13]

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It is often stated without much evidence that UVA is more important than UVB for photoageing as it penetrates deep into the dermis, unlike UVB. Such statements ignore the possibility that epidermally derived factors may influence dermal ECM, and indeed there is evidence that cytokines/TNFα, readily induced by UVR,[14,15] can induce MMP synthesis.[16,17] Furthermore, there have been very few studies that have investigated UVR penetration of the skin, and studies that have done this have used physical, rather than biological techniques, often on disrupted isolated skin.[18,19]

There are no human data on the action spectrum for photoageing. Mouse studies[20] have suggested that the action spectrum for elastosis, a hallmark of photoageing, is similar to that for erythema, in which UVB is 3–4 orders of magnitude more effective than UVA. Chronic UVA exposure in hairless mice is capable of inducing photodamage like UVB[21] and, given the high quantity of UVA in sunlight, there is reason to believe that UVA could make a significant contribution to photoageing.[22] One study evaluated skin cancer and elastosis in mice exposed to narrow band UVB and UVA;[20] UVB induced SCC and elastosis whereas UVA induced elastosis only but was less effective than UVB.

The aim of this study was to test the hypothesis that UVR-induced erythema and MMP-1 mRNA induction share a common action spectrum. We approached this by giving exposures from 3 UVR sources that resulted in equal erythemal responses observed at 24 h after irradiation. The sources were UVB (300 nm), UVA1 and solar simulated radiation (SSR). If our hypothesis were correct, we would predict comparable MMP-1 mRNA expression with comparably effective erythemal exposures. In this context we also assessed the expression of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1).

A common action spectrum for erythema and MMP-1 mRNA induction would suggest common chromophores. There is indirect human *in vivo* evidence that epidermal DNA is an important chromophore for erythema,[2] and *in vitro* and *in vivo* evidence that DNA is a chromophore for the induction of MMP-1 mRNA and protein.[23] UVR-induced MMP-1 is expressed in the epidermis and dermis at the mRNA and protein level.[11,12] It is not known whether the dermal mRNA induction is due to factors released by the epidermal cells, or the result of direct UVR effects on dermal cells, such as fibroblasts. Little is known about dermal DNA photodamage so we also investigated the effects of UVA1 and UVB on the induction and location of dermal T<>T and its repair.

Results

UVA1, UVB and SSR induce comparable expression of MMP-1 mRNA

Fig. 1 shows the induction of mRNA MMP-1 expression by (1a) UVA1 and (1b) UVB. A linear regression model showed substantial dose-response effects for UVB and UVA1 and a substantial subject level effect as can seen by the large inter-person variation in the figures. Overall, the increase is 80.6 times higher (compared to baseline) with UVA1 and 34.3 times higher with UVB, giving a ratio of 2.35 (95% CI 0.44 to 12.6, $p = 0.32$) which is not significantly different from 1. A non-linear model that we used also allowed for arbitrary non-linear effects of dose and this indicated a threshold of 1 MED for

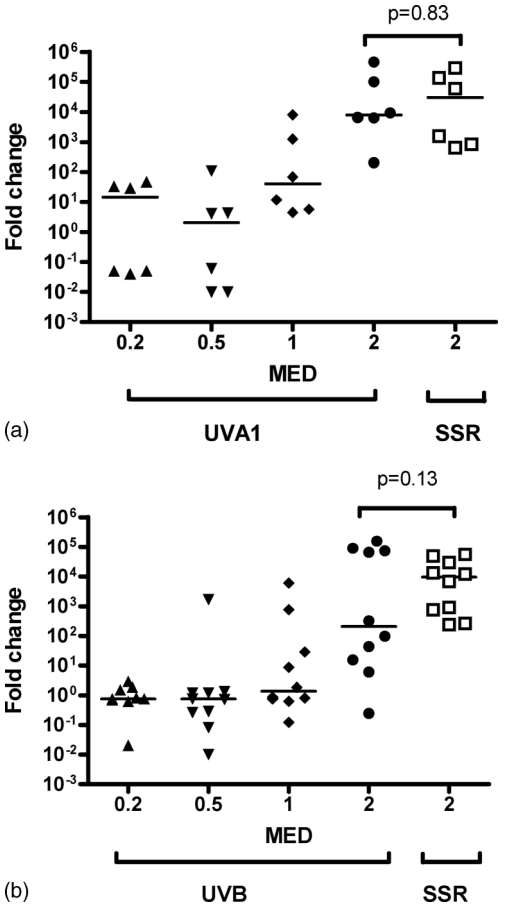

Fig. 1 Effect of (a) UVA1 and (b) UVB on MMP-1 mRNA expression in comparison with 2MED SSR. The horizontal bars represent median values. A linear regression model showed a highly significant effect of UVA1 and UVB dose, but that these dose-response curves were not significantly different from each other.

a significant effect for both spectra. The level of SSR-induced mRNA expression is comparable in the two groups of volunteers ($p = 0.1$) and comparisons of 2 MED SSR, UVB and UVA1 showed no differences ($p > 0.1$).

UVA1 induces more TIMP-1 mRNA expression than UVB

Fig. 2 shows the induction of mRNA TIMP-1 expression by (2a) UVA1 and (2b) UVB. The induction of TIMP-1 is much lower than for MMP-1, as we have previously reported for SSR.[12] The linear model showed a significant effect of dose for UVB and UVA1 but there was no subject level effect. The overall increase for UVA1 was 2.2 and 1.5 for UVB from baseline, giving a ratio of 1.48 (95% CI 1.09–2.01, $p = 0.01$), which means that UVA1 is significantly more effective than UVB at inducing TIMP-1 expression. The level of SSR-induced mRNA expression is comparable in the two groups of volunteers ($p = 0.08$). There is no difference between 2MED UVA1 and 2 MED SSR ($p = 0.59$), but significantly more TIMP-1 was induced with 2MED SSR compared with 2 MED UVB ($p = 0.03$). The dose-response results and those from the 2 MED comparisons are consistent given that UVA1 is the major component of SSR.

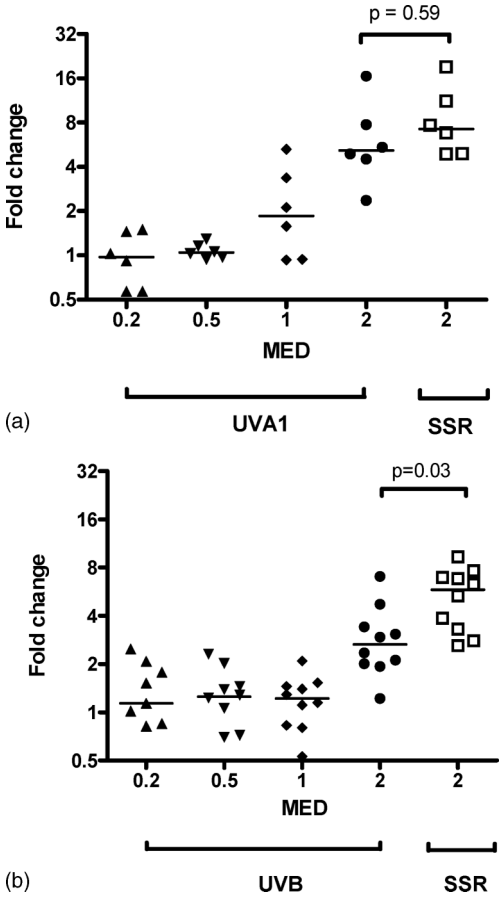

Fig. 2 Effect of (a) UVA1 and (b) UVB on TIMP-1 mRNA expression in comparison with 2 MED SSR. The horizontal bars represent median values. A linear regression model showed a highly significant effect of UVA1 and UVB dose, with UVA1 being more effective than UVB.

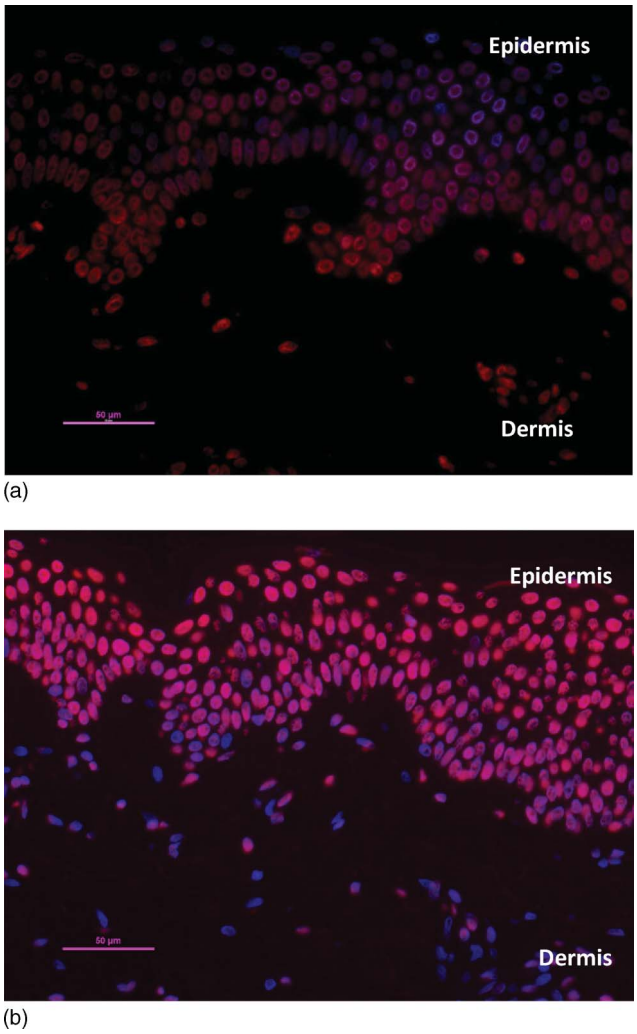

Fig. 3 Staining of dermal nuclei for T<>T, 3 notional MED (a) UVA1 (b) UVB. The red stain is indicative of T<>T. Biopsies were taken immediately after exposure. See Tewari *et al.*[24] for quantification of epidermal data that allows comparisons with Fig. 4, 5 and 6.

The effect of dermal depth on UVA1 and UVB-induced T<>T staining

Examples of epidermal and dermal T<>T staining for UVA1 and UVB are shown in Fig. 3a and 3b, respectively. Fig. 4a and 4b show the relationship between T<>T staining and depth from the basal epidermis for UVA1 and UVB, respectively. The data show the degree of T<>T is independent of depth ($p = 0.53$ for slope) for UVA1, whereas there is a highly significant ($p = 0.0001$ for slope) loss of stain with depth with UVB.

Dose response and repair kinetics for dermal T<>T with UVA1 and UVB

Fig. 5 shows a dose-dependent increase in T<>T for UVA1 (slope $p = 0.009$) and UVB (slope $p < 0.0001$). The maximal damage with UVB is about 3 fold greater that UVA1, which is similar to what we have reported in the epidermis.[24] Fig. 6 shows that repair of dermal T<>T is slow. The slope for UVA1 is significant ($p = 0.03$) but that for UVB is not significant ($p = 0.62$).

Discussion

We have previously shown that SSR induced MMP-1 mRNA in a dose dependent way with maximal expression at 24 h;[12] with high levels following 2 MED. In contrast, the effect on TIMP-1 was very modest. Comparable levels of mRNA expression of MMP-1 were seen in the epidermis and dermis with SSR, whereas TIMP-1 was only seen in the dermis.[12]

In the current study, our results are for mRNA only, but other groups have shown that UVB also induces MMP-1 protein and its activity in the dermis and epidermis.[10,11] We used 2MED SSR as a positive control in UVB and UVA1 dose-response studies with comparable erythemal exposure. Our data show considerable inter-personal variation in MMP-1 mRNA expression, which has been reported by us[12] and others,[23] one reason for which may be different single-nucleotide polymorphisms (SNPs) that may influence gene transcription.[25] However, the dose-response curves (as MED fractions) for UVB and UVA1-induced MMP-1 were not significantly different, and there was no statistical difference between MMP-1 mRNA expression levels with 2MED by three different spectra. This provides evidence that the action spectra for erythema and MMP-1 mRNA induction are the same or very similar. However, it should be noted that the confidence limits on

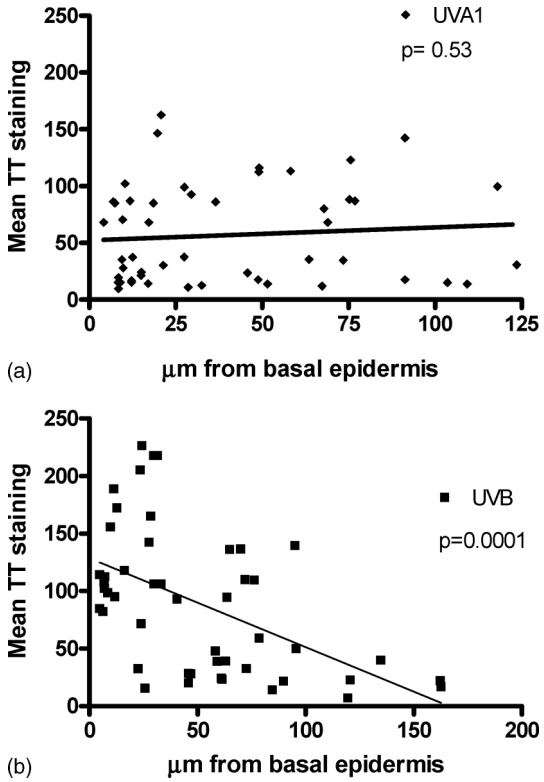

Fig. 4 Influence of dermal depth on T<>T staining after 3 notional MED (a) UVA1 and (b) UVB. A total 50 nuclei for each spectrum were examined from 12 volunteers. Biopsies taken immediately after exposure and the data have been analyzed by linear regression and p values refer to slopes of regression lines. The mean T<>T staining (vertical axis) represents the "red intensity" of each nucleus. This is calculated from the average intensity of each pixel within a nucleus and corresponds to the amount of T<>T present.

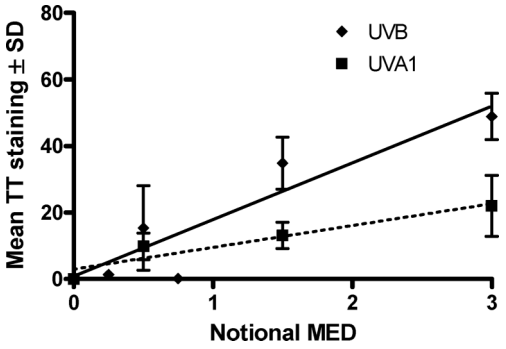

Fig. 5 Dose response for dermal T<>T induced by UVA1 and UVB. Both slopes are significant ($p < 0.01$). The mean T<>T staining (vertical axis) represents the average "red intensity" of nuclei of a given volunteer at a given dose.

which this conclusion is based are high, due to large inter-person variability.

This similarity of action spectra suggests common chromophores. Two main photochemical events have been proposed for the induction of MMP-1: i) the formation of cyclobutane pyrimidine dimers (CPD)[23] and ii) UVR induced reactive oxygen species (ROS), primarily by UVA *via* chromophores that have yet to be identified.[26] Until recently, DNA was primarily regarded as

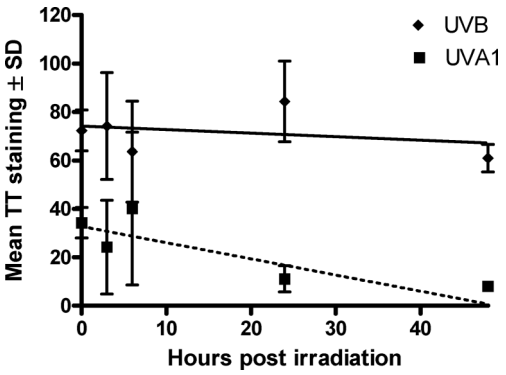

Fig. 6 Repair of dermal T<>T after exposure to 3 notional MED UVA1 and UVB. The slope (p 0.03) of UVA1 is significant, but this is not the case for UVB ($p = 0.62$). The mean T<>T staining (vertical axis) represents the average "red intensity" of the nuclei of a given volunteer at a given post-irradiation time.

a UVB chromophore, but studies show that UVA1 induces more CPD, especially T<>T, than expected.[27,28] To date, the focus on UVA has primarily been on its ability to generate ROS. This yields oxidized bases, such as 8-oxo-7,8-dihydro-guanine (8-oxoGua), and DNA strand breaks.[29] DNA, resulting in the formation of CPD, has been proposed as an important chromophore for erythema[2] and TNFα[15] (which may initiate MMP-1 induction). Laser action spectroscopy for human erythema has identified a minor independent UVA1 absorbing chromophore.[30] UVB-induced erythema is oxygen independent but UVA1 induced erythema requires oxygen.[31] Overall, these data suggest a role for ROS in UVA1-induced erythema. Thus, our MMP-1 data are consistent with the hypothesis that DNA and an unknown UVA1 absorbing molecule, that probably generates ROS, are shared chromophores for erythema and MMP-1 induction. Support for an important role for DNA comes from human skin *in vivo* studies in which skin was exposed to 1 MED UVB. Post-irradiation treatment with an activated CPD photolyase reduced MMP-1 mRNA expression as well as epidermal and dermal MMP-1 protein visualized by immunohistology.[23]

Interpreting action spectroscopy in the skin is difficult because UVR penetration is wavelength dependent and the location of the chromophore and the biological outcome of its activation may be different. Erythema and collagen degradation by MMP-1 are both dermal events that are possibly initiated by photochemical activity in the epidermis; for example, the transfer of media from UVB-irradiated human keratinocytes induces MMP-1 mRNA and protein expression in unirradiated human fibroblasts.[23] The dose-range that we used in the MMP-1 studies readily induces dermal T<>T, especially with UVB as shown in Fig. 5, but Fig. 3 and 4 show that the location of the damage is spectrally dependent. UVB-induced lesions are focused in the upper dermis whereas UVA1 induced lesions, although fewer in number (characterised by less red intensity), are evenly spread throughout the dermis. Thus, our data suggest that direct dermal UVR-induced expression of MMP-1 mRNA is possible. Enhanced repair of CPD in skin cells *in vitro* and *in vivo* has been shown to inhibit MMP-1 mRNA and protein.[23] We have previously shown that epidermal repair of SSR and UVA1-induced T<>T is slow [24,32,33] and Fig. 6 shows that this is also the case in the dermis; indeed there was no evidence for the

repair of UVB-induced lesions. Thus persistent lesions may well initiate dermal MMP-1 expression.

As with SSR, the effects of UVB and UVA1 on TIMP-1, which is only expressed in the dermis, are relatively modest.[12] However, there was a significant dose-response with UVB and UVA1 but the effects of the latter were significantly greater than the former. In addition, 2 MED SSR was significantly more effective than 2 MED UVB, whereas the effects of 2 MED UVA1 and 2 MED SSR (mostly UVA1) were the same. One reason for the greater efficacy of UVA1 may be its better penetration into the dermis. The relationship between UVR-induced MMP-1 and TIMP-1 mRNA and protein/activity is not known.

It is often stated that UVA is the main cause of photoageing. However, irrespective of chromophores, if the SSR spectrum that we used is weighted with the CIE action spectrum for erythema,[34] we can state that 87% of MMP-1 induction is due to UVB (defined as 280–320 nm). A comparable weighting for London (51°N) noon summer sun would result in 80% of MMP-1 induction being due to UVB. Even assuming that our hypothesis is false, and that UVA1 is 2.35 times more effective than UVB at comparable erythemal exposures, we would still conclude that solar UVB is the main cause of photoageing. These conclusions are based on acute exposures and do not account for any photoadaptation, which has been reported after repeat exposure studies for some endpoints.[35] An action spectrum for solar elastosis in hairless mice was also similar to that for human erythema,[36] which would also implicate UVB as the most important spectral region in practice. Most action spectra for photoageing markers in mice have shown UVB to be more important than UVA apart from skin "sagging".[22]

Sunscreens inhibit erythema, as measured by their sun protection factors (SPF). Comparable SPFs can be obtained with different ratios of UVB:UVA protection, which can predict protection from SSR-induced epidermal T<>T.[37] Our results suggest that the inhibition of erythema by sunscreen use of a given labelled SPF should comparably inhibit MMP-1 expression. However, we have also reported that SPF in practice will decrease with lower solar UVB content (dependent on latitude, time of day, *etc*) with a primarily UVB sunscreen.[38] This is not the case with broad spectrum sunscreens in which actual SPF is independent of solar UVB content. Overall, it is better to have a robust index of protection, and we therefore advocate the use of broad spectrum protection with comparable levels of UVB and UVA protection.

Conclusions

Although we have not done a conventional action spectrum study with dose-response curves at different monochromatic wavelengths, our analyses suggests that the action spectra for erythema and acute MMP-1 mRNA expression are the same. This suggests common chromophores and we propose DNA and an unknown UVA1 absorber that may mediate its effects by ROS. In contrast, the action spectra for erythema and TIMP-1 mRNA expression are different, with UVA1 being relatively more effective than UVB. This could mean that the chromophores for MMP-1 and TIMP-1 are different, but it could also reflect the different location of the chromophores within the skin.

Maximal ambient exposure during a clear sky summer's day in the UK is about 45 standard erythema doses (SED), of which the UVA1 contribution is approximately 7%; equivalent to 3 SED or ~1 MED in a skin type I/II person.[39] Thus, assuming that the action spectra for erythema and MMP-1 induction are similar, the UVA1 contribution to photoageing in practice is likely to be minor. We can conclude that solar UVB is the main cause of photoageing.

Experimental

Volunteers

The studies were approved by the St Thomas' Hospital, London, UK Ethics Committee (Ref: EC00/006 and Ref: 09/H0802/98)

Table 1 Volunteer demographics and just perceptible MED for MMP-1/TIMP-1 studies. 95.1% of the EEE of the UVA1 source was UVA1, 4.1% UVA2 and 0.8% UVB. In the case of SSR 87.1% EEE was UVB, 5.1% UVA1 and 7.8% UVA2. There was no significant difference in age ($p = 0.10$) or SSR MED ($p = 0.10$) between the UVA1 and UVB groups. 4(25%) volunteers were smokers, but this did not have an impact on control (*i.e.* no UVR) MMP-1 expression ($p = 0.17$)

Study	Skin type	Sex	Age	SSR MED (J cm^{-2})	UVA1 MED (J cm^{-2})
UVA1 and SSR	I	F	19	5.1	100
	I	F	33	2.9	25
	I	M	36	4.9	70.7
	II	F	33	5.8	50
	I	M	35	4.9	35.4
	II	F	19	5.1	100
Totals and mean ± SD	**4I + 2II**	**2M + 4F**	**29.3 ± 7.7**	**4.8 ± 0.97**	**63.5 ± 32.2**
Study	**Skin type**	**Sex**	**Age**	**SSR MED (J cm^{-2})**	**UVB MED (mJ cm^{-2})**
UVB and SSR	II	M	26	6.1	14
	II	M	22	6.1	20
	II	F	22	6.1	28
	I/II	M	22	3.9	28
	II	M	27	4.9	40
	I/II	M	24	6.1	14
	II	M	**31**	5.3	**40**
	II	F	**29**	3.9	**28**
	II	F	**21**	4.9	**20**
	II	M	**20**	3.9	**14**
Totals and mean ± SD	**2I/II + 8II**	**7M + 3F**	**24.4 ± 3.7**	**5.1 ± 0.98**	**24.6 ± 9.9**

Table 2 Volunteer demographics and just perceptible MED for dose-response and time-course T<>T studies. 99.3% of the erythemally effective energy (EEE) of the UVA1 source was UVA1 and 0.7% was UVA2. There was no significant different in age ($p = 0.89$) between the two study groups. The mean UVA1 MED in the dose-response group was higher than in the time-course group ($p = 0.03$) but there was no difference in the UVB MED ($p = 0.40$)

Study	Skin type	Sex	Age	UVA1 MED (J cm^{-2})	UVB MED (mJ cm^{-2})
Dose-response	I	F	28	48.8	30.0
	I	F	25	48.8	30.0
	II	M	20	48.8	30.0
	II	F	21	53.0	30.0
	I	F	22	48.8	23.0
	I	M	34	61.1	19.0
Totals and mean ± SD	4I + 2II	2M + 4F	**25 ± 5.3**	**51.6 ± 4.9**	**27.0 ± 4.8**
Time-course	I	F	28	31.3	19
	I	F	28	31.3	30
	I	M	22	48.8	23
	II	F	24	31.3	30
	I	M	23	48.8	23
	II	F	23	48.8	23
Totals and mean ± SD	4I + 2II	2M + 4F	**24.7 ± 2.7**	**40.1 ± 9.6**	**24.7 ± 4.4**
Combined	8I + 4II	4M + 8F	**24.8 ± 4.0**	**45.8 ± 9.4**	**25.8 ± 4.6**

and done in accordance with the declaration of Helsinki. Normal healthy skin type I–II young adults gave written informed consent before taking part. The demographic details of the 16 volunteers for the MMP-1/TIMP-1 studies and the 12 volunteers for the T<>T studies are similar and are shown in Tables 1 and 2, respectively.

UVR sources and dosimetry

The emission spectra of the UVR sources are shown in Fig. 7 (UVB1 source for MMP-1/TIMP-1 data, UVB2 for T<>T data). These were determined with a DM150BC double-monochromator spectroradiometer (Bentham Instruments, Reading, UK) using an integration sphere and gratings blazed at 250 nm. The spectroradiometer was calibrated against national UK standards.

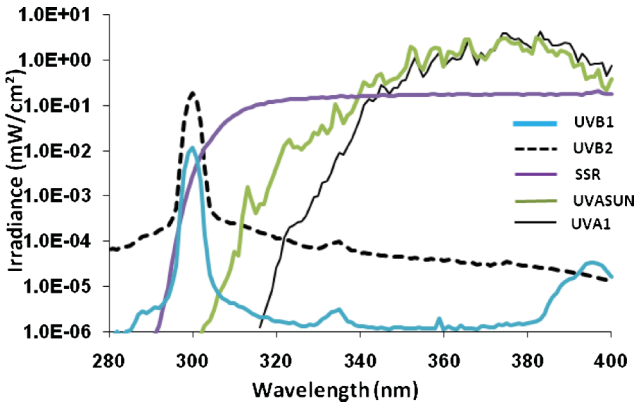

Fig. 7 Emission spectra of the sources used. UVB1 corresponds to the spectrum for Fig. 1–2 and UVB2 corresponds to the spectrum for Fig. 3–6.

SSR was produced by an Oriel solar simulator (Model 81292, L.O.T Oriel, Leatherhead, UK) with a 1-kW xenon arc lamp with two dichroic mirrors, a collimator, and a 1-mm WG320 filter. Irradiance was routinely measured by a wide-band thermopile radiometer (Medical Physics, Dryburn Hospital, Durham, UK) that had been calibrated against the spectroradiometric measurements. This was typically 15 mW cm^{-2} at skin surface (11 cm from source).

Two UVA1 sources were used. For the MMP-1/TIMP-1 studies, this was a UVASun 2000 (Mutzhas, Munich, Germany). Routine dosimetry was done with a radiometer (Model IL442A (with a UVA sensor), International Light technologies, Massachusetts, USA), after calibration against the spectroradiometric measurements. Irradiance was 70–75 mW cm^{-2} at the skin surface (11 cm from the source). For the T<>T studies, this was a Sellamed 3000 Dr Sellmeier (Sellas, Gevelsberg, Germany) phototherapy device. Irradiance was routinely measured with a radiometer (Model IL1400A, International Light technologies, Massachusetts, USA), after calibration against the spectroradiometric measurements, and was typically ~74 mW cm^{-2} at skin surface (24.5 cm from source).

Narrowband UVB (300 nm) was produced by a monochromator (L.O.T. Oriel, Newport, USA: 1-kW xenon arc; grating blazed at 250 nm; slits set for 3 nm full width at half maximum (FWHM) bandwidth) and 2.5 nm FWHM UVB was delivered with a liquid light guide (Oriel), exit diameter 5 mm, in direct contact with the skin. In the studies described in Table 1, irradiance (typically 0.3mW cm^{-2}) was measured with a wide-band thermopile radiometer (Medical Physics, Dryburn Hospital, Durham, UK). In the case of studies in Table 2, irradiance (typically ~0.5 mW cm^{-2}) was measured with an SEL623 thermopile (International Light Technologies, Massachusetts, USA) attached to an IL1400A photometer calibrated by the United Kingdom Accreditation Service-accredited Guy's and St Thomas' Hospitals Trust UVR laboratory.

Irradiation protocol

A geometric ($\times\sqrt{2}$ for the MMP-1/TIMP-1 study and $\times 1.25$, for the T<>T study) series of doses was given over 1 cm^2 areas of previously unexposed buttock skin or over the 5 mm exit diameter of the liquid light guide in the case of 300 nm. A visual assessment of the exposed sites was made 24 h after exposure and the 'just perceptible MED' was determined as it is a more reliable threshold endpoint than 'erythema with a definite border'.[40]

Previously unirradiated buttock skin of the MMP-1/TIMP-1 study volunteers (Table 1) was given 0.2, 0.5, 1 and 2 MED of UVB or UVA and 2 MED of SSR, based on their individual

MED and 4 mm punch biopsies were taken 24 h after exposure. This time was based on our previous time-course studies with SSR for MMP-1 and TIMP-1.[12]

The dose protocols in the T<>T studies were based on "notional MED". These were derived from the MED from 3 individuals: mean values 48.8 J (SD ± 0) cm^{-2} for UVA1 and 30.0 (SD ± 0) mJ cm^{-2} for UVB and the mean MEDs given in Table 2 were within 1–2 SD of these values. Table 2 shows the demographics of the volunteers in the dose response and repair studies. In the former, 6 volunteers were given 0.5, 1.5 and 3 notional MED of UVB and UVA1 on previously unexposed buttock skin and biopsies were taken immediately afterwards. In the latter, 6 volunteers were given 3 notional MED of UVA1 and UVB and biopsies taken at 3 time-points from: immediate, 3, 6, 24 and 48 h (times varied from person to person because of ethical restrictions: all had biopsies at 0 and 24 h, except for 2 UVB sites on 2 volunteers. A non-irradiated control biopsy was taken from all 12 volunteers).

Immunostaining and image analysis

Full details are given in our previous studies.[24] Briefly 5 μm paraffin sections were stained with the monoclonal antibody incubation: CPD TDM-2 (CosmoBio, Tokyo, Japan) at 1 : 2000 (antibody specificity to UVR induced T<>T [41]) and isotype control IgG2a (DAKO, Cambridge, UK) for 90 min at room temperature. Fluorescent imaging was done for AlexaFluor555 (red) and DAPI (nuclear DNA stain; blue) with a Zeiss Axiophot microscope (Harpenden, UK), Nikon DS-U2 camera (Kingston-upon-Thames, UK) with ×20 magnification (and ×1.25 eyepiece objective). Using NIS elements BR v3 software package, images acquired in 2560 × 1920 format and exposed to 20S (gain 1.40× for AlexaFluor555 with 1.5 s exposure, gain 1.40× for DAPI). The contrast setting was the same for all images. DAPI stained nuclei were gated and mean red intensity (Alexa Fluor 555) assessed within each of the nuclei in the dermis. Mean background intensity from the unirradiated control was subtracted from the irradiated samples to control for nonspecific nuclear staining (typically for T<>:T: 10.0 ± 6.4(SD) as previously reported[33]). Settings for nuclei capture (circularity and diameter of nuclei) were kept constant, and sections subjectively assessed. It was not possible to stain and assess all slides in a single batch. To assess the reproducibility of the techniques, the same positive controls (3 MED UVB and UVA1) were processed with each staining and image analysis run, as well as a non-irradiated control and an isotype control in each case.

RT-PCR

We used the TaqMan™ method, with probes for MMP-1, TIMP-1 and GAPDH (housekeeping), as described in detail in our previous studies.[12] RNA was extracted from whole skin 4 mm punch biopsies. The raw C_T data were converted using the comparative $\Delta\Delta C_T$ method.

Statistics

Statistical analyses were done using Stata (StataCorp LP, College Station, Texas) using a mixed-effects random effects maximum likelihood model (REML) allowing for grouping by subject. Log_{10} gene expression data were analysed using a simple linear regression dose response to UVB and UVA1, as well as a non-linear model. The latter allowed for possible arbitrary non-linear effects of dose by fitting dummy variables to account for the four-dose (MED fraction) levels. The two models gave very similar results, so unless otherwise stated the results are based on the linear model. Graphpad Prism v4 was used for two-tailed paired and unpaired (as appropriate) T tests to compare volunteers' ages, MED and MMP-1 and TIMP-1 mRNA expression. In the case of data from Fig. 1, 2 MED UVB and 2 MED UVA were compared by using the individual volunteer SSR Δ values. Graphpad Prism v4 was also used for linear regression analysis of the T<>T data.

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UVA1 Induces Cyclobutane Pyrimidine Dimers but Not 6-4 Photoproducts in Human Skin *In Vivo*

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UVB readily induces cyclobutane pyrimidine dimers, mainly thymine dimers (TTs), and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) in DNA. These lesions result in "UVB signature mutations" found in skin cancers. We have investigated the induction of TTs and 6-4PPs in human skin *in vivo* by broadband UVA1, and have compared this with comparable erythemal doses of monochromatic UVB (300 nm). *In vitro* and *ex vivo* studies have shown the production of TTs, without 6-4PPs, by UVA1. We show that UVA1 induces TTs, without 6-4PPs, in the epidermis of healthy volunteers *in vivo*, whereas UVB induced both photoproducts. UVB induced more TTs than UVA1 for the same level of erythema. The level of UVA1-induced TTs increased with epidermal depth in contrast to a decrease that was seen with UVB. UVA1- and UVB-induced TTs were repaired in epidermal cells at a similar rate. The mechanism by which UVA1 induces TTs is unknown, but a lack of intra-individual correlation between our subjects' UVB and UVA1 minimal erythema doses implies that UVA1 and UVB erythema occur by different mechanisms. Our data suggest that UVA1 may be more carcinogenic than has previously been thought.

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INTRODUCTION

Solar UVR is the main cause of skin cancer (Armstrong and Kricker, 2001), and UVB (290–320 nm) has generally been thought of as the major carcinogen. However, UVA (320–400 nm) is 20 times more abundant than UVB in terrestrial UVR, and around 75% of solar UVA is UVA1 (340–400 nm). On average, an indoor worker is exposed to 1,500 J cm^{-2} UVA exposure per year (Diffey, 1996). UVA is the predominant waveband emitted by sunbeds despite recent classification as a carcinogen to humans (El Ghissassi *et al.*, 2009), and UVA1 is increasingly used for phototherapy for skin diseases (Dawe, 2003; York and Jacobe, 2010).

Although the action spectrum for induction of squamous cell carcinoma in mice has a major UVB peak, there is also a smaller peak in the UVA1 region (de Gruijl *et al.*, 1993). It has been reported that UVA causes melanoma precursors in *Monodelphis domestica* (Ley, 1997), and melanoma in *Xiphophorus* fish (Setlow, 1974; Setlow *et al.*, 1993), but this has been recently refuted (Mitchell *et al.*, 2010). A role for UVA in human skin cancer has been proposed (Drobetsky *et al.*, 1995; Agar *et al.*, 2004), which is supported by some epidemiological data for melanoma (Garland *et al.*, 2003).

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Abbreviations: 6-4PP, pyrimidine (6-4) pyrimidone photoproduct; CPD, cyclobutane pyrimidine dimer; TT, cyclobutane thymine dimer

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DNA absorbs UVB, and UVA to a smaller degree (Setlow, 1974; Sutherland and Griffin, 1981; Young *et al.*, 1996). Absorption of UVB causes the formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). These lesions are repaired by the excision repair pathway, but when unrepaired can form the classic ''UVB signature'' mutations: C→T or CC→TT (Ziegler *et al.*, 1996), which are found in skin cancers.

UVA is also mutagenic, but its genetic effects have been mainly attributed to UVA excitation of non-DNA chromophores, resulting in reactive oxygen species-induced base oxidation to form products such as 8-oxo-7,8-dihydroguanine, as well as DNA single-strand breaks (Cadet *et al.*, 2009). UVB also induces 8-oxo-7,8-dihydroguanine (Ravanat *et al.*, 2001).

Unexpectedly, studies in cultured cells and whole explant skin have shown that CPDs are induced by UVA1 in larger amounts than oxidatively generated lesions (Douki *et al.*, 2003; Mouret *et al.*, 2006). In addition, the classic CPD-induced ''UVB signature'' mutation C:G→T:A has been found to be the most common mutation induced by UVA (41% of mutations; Rochette *et al.*, 2003; Kappes *et al.*, 2006). This has raised doubts about the relative mutagenicity and carcinogenicity of UVA and UVB in human skin as the ''UVB signature'' mutations found in skin cancers, including melanoma, may also be caused by UVA.

Interestingly, unlike UVB, UVA1 produces cyclobutane thymine dimers (TTs) *in vitro* and *ex vivo* but few CC, CT, and no 6-4PP or their Dewar isomers (Douki *et al.*, 2003; Mouret *et al.*, 2006). An indirect photosensitized triplet energy transfer mechanism with UVA1 was originally suggested to explain this (Cadet *et al.*, 2009), but this is no longer thought

to be the case (see discussion). UVA1 induction of CPDs has been studied *in vitro* and *ex vivo* (Mouret *et al.*, 2006). However, the important issue of the role of UVA1 in CPD formation *in vivo* has not been studied.

We therefore aimed to measure the formation of TTs and 6-4PPs in humans *in vivo* by comparable erythemal doses of UVA1 and UVB (300 nm). Erythema was used as the comparator because it is a key response to UV damage, and human action spectroscopy has specifically implicated TT formation as a trigger for erythema (Young *et al.*, 1998). In addition to induction, we assessed the repair of photoproducts with UVA1 and UVB *in vivo*, because it has been reported that TT repair is slower *ex vivo* after UVA1 than UVB, which is relevant to the formation of mutations (Mouret *et al.*, 2006).

RESULTS

Erythema

Comparable levels of erythema were achieved for the same multiple of the notional minimal erythema dose (MED; see Material and Methods for definition) for both UVB and UVA1 (Figure 1a). This is a fixed physical dose based on the mean MED of three individuals (see Materials and Methods). The slope for UVB was steeper than that for UVA1 ($P = 0.0006$); there was no correlation between a given individual's UVB and UVA1 MED (Figure 1b).

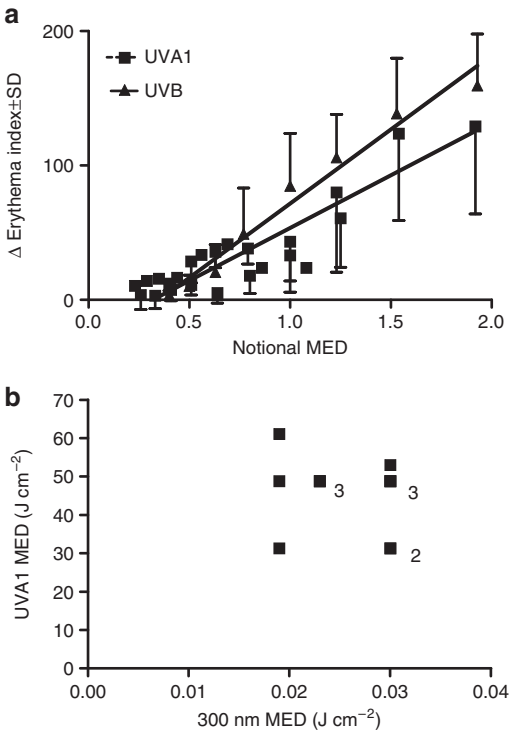

Figure 1. UVA1- and UVB-induced erythema. (**a**) Quantitative erythema dose responses for UVB (300 nm) and UVA1. These data were obtained from the individual MED assessments series and are plotted against notional MED fractions. The slopes for both spectra are highly significant ($P < 0.001$). (**b**) Lack of relationship between a given individual's UVB and UVA1 MED, $n = 12$: note numbers on the figure correspond to the number of volunteers with the same UVB and UVA MEDs. MED, minimal erythema dose.

Epidermal localization of TT and quantification of depth of staining

Figure 2a and b shows anti-TT antibody staining after UVA1 and UVB, immediately after exposure to three notional

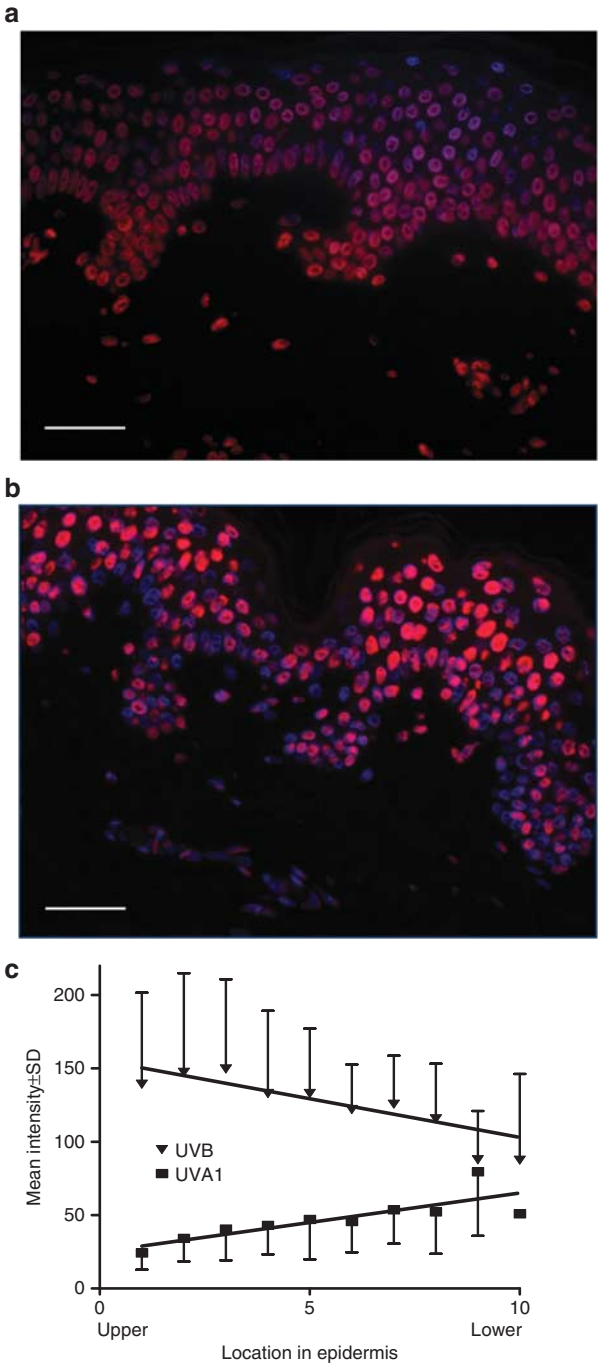

Figure 2. Immunostaining of UV-irradiated epidermis. Typical microscope images of (**a**) UVA1 and (**b**) UVB assessed immediately after exposure to three notional MEDs. Note more intense red (cyclobutane thymine dimer, TT) staining at basal epidermis with UVA1 and more intense red staining in upper epidermis with UVB. (**c**) Quantification of TT staining on a nucleus-per-nucleus basis with epidermal depth for 12 volunteers. The lines were generated by linear regression and both slopes were significant ($P \leqslant 0.01$). Bars = 50 μm (**a**) and 50 μm (**b**). MED, minimal erythema dose.

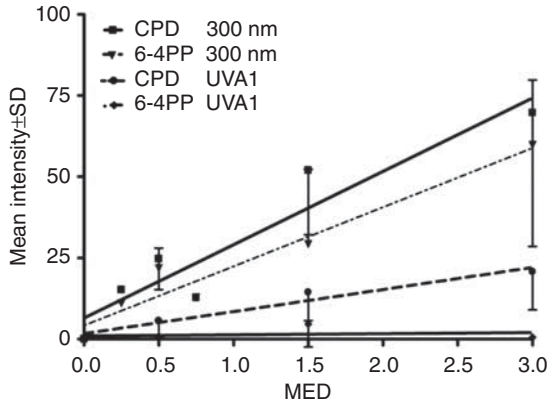

Figure 3. Dose responses for cyclobutane thymine dimer and 6-4PP with UVB (300 nm) and UVA1. The lines were generated by linear regression. Note: in one case the volunteer was inadvertently given a different dose series. 6-4PP, pyrimidine (6-4) pyrimidone photoproduct; CPD, cyclobutane pyrimidine dimer; MED, minimal erythema dose.

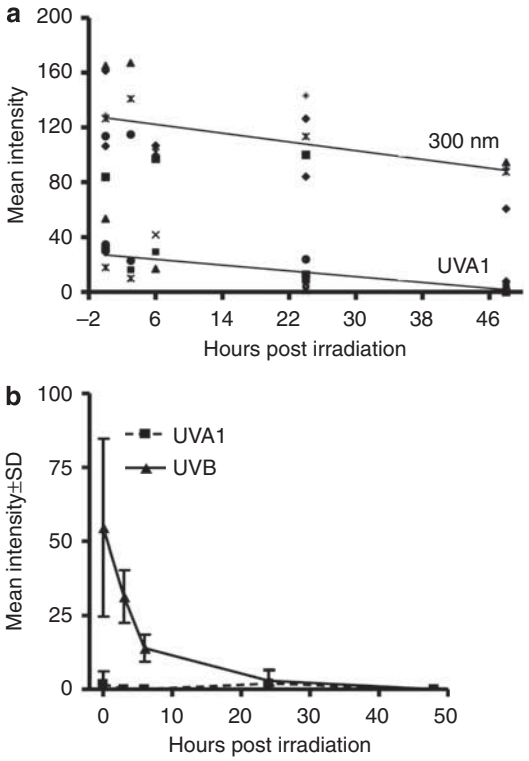

Figure 4. Repair of epidermal photolesions. (**a**) Repair kinetics of cyclobutane thymine dimer and (**b**) pyrimidine (6-4) pyrimidone photoproduct after three notional MEDs UVB (300 nm) and UVA1. The lines were generated by linear regression and the slopes are not significantly different ($P=0.71$).

MEDs. All isotype controls were negative (data not shown). UVA1 caused more intense staining in the lower epidermis, whereas UVB led to more intense upper epidermal staining. We quantified the relationship between staining intensity and epidermal depth by linear regression analysis to a depth of 10 nuclei (Figure 2c, $n=12$). With UVB there was a significant ($P=0.01$) reduction in TT staining with increased epidermal depth. In contrast, for UVA1, there was a significant ($P<0.001$) increase in staining with increased depth.

Dose dependence of photoproduct formation

For a given multiple of notional MED, UVA1 produced fewer TTs than UVB (Figure 3): the slope for UVB is 3.4-fold steeper than that with UVA1 ($P=0.00096$). Immediately after exposure to UVB, 4.4 times more TTs were found than with a comparable dose of UVA1 (Figure 4a). 6-4PPs are induced by UVB in a dose-dependent manner but no 6-4PPs at all are induced with UVA1 at any dose *in vivo* (Figure 3).

Repair kinetics of CPD and 6-4PP

The slopes for the loss of UVB- and UVA1-induced TTs with time are not significantly different ($P=0.71$). By 48 hours, the majority of the UVA1-induced TTs had disappeared (Figure 4a). However, UVB-induced TTs are still present because 3MED UVB induces more TTs than 3MED UVA1. Repair of UVB-induced 6-4PPs was very fast (Figure 4b), as we previously reported *in vivo* (Young *et al.*, 1996; Bykov *et al.*, 1999) for UVB using solar-simulated radiation.

The comparable repair rate for TTs induced by both spectra suggests that the same host repair machinery is used. Our data are different from published *ex vivo* studies (Mouret *et al.*, 2006) that suggest that UVB-induced TTs were repaired faster than those induced by UVA1.

DISCUSSION

We have shown that UVA1 readily induces TTs but not 6-4PPs in human skin *in vivo*. To our knowledge this is previously unreported in humans *in vivo*, as is the comparison of the effects of UVA1 to erythemally equivalent doses of UVB. Our maximum doses for UVB (300 nm) and UVA1 were 90 mJ cm^{-2} and 148 J cm^{-2} compared with 20 mJ cm^{-2} and 200 J cm^{-2}, respectively, used in the previous study using *ex vivo* skin (Mouret *et al.*, 2006). As the UVB in that study was primarily at 312 nm, those exposures would have been sub-erythemal as MEDs for skin type I/II at 312 nm are 200–300 mJ cm^{-2} (Palmer *et al.*, 2006); thus, previous *ex vivo* studies have not used UVA1 and UVB doses with comparable erythemal potential. Erythema is widely accepted as the biologically relevant measure of acute UVR exposure (e.g., a sunscreen's sun protection factor is measured by its protective effect against erythema). Our data show comparable levels of erythema for UVA1 and UVB for a given notional MED fraction (Figure 1a), although the dose response for UVB is significantly steeper. The absence of any correlation (Figure 1b) between an individual's UVB and UVA1 MED suggests that UVA1 erythema may involve a different mechanism. Epidermal DNA is thought to be an important chromophore for UVB erythema induction (Young *et al.*, 1998). When it produces approximately the same intensity of erythema, UVA1 induces 3- to 4-fold fewer TTs than UVB. This implies that other chromophores may be important for UVA1 erythema. The second UVA1 peak identified in one action spectrum of human erythema also points to the

chromophores possibly being different for UVA1 (Anders *et al.*, 1995). UVB-induced erythema is oxygen independent, whereas UVA erythema is oxygen dependent (Auletta *et al.*, 1986), implying a role for reactive oxygen species in UVA erythema.

The majority of CPDs induced by UVA1 are TTs (Mouret *et al.*, 2006), and we show the presence of UVA1-induced TTs using an antibody technique, although it is possible that some cytosine-containing CPDs were also induced because the antibody is not 100% TT specific (see Materials and Methods). TTs are much less mutagenic than CC and CT (Pfeifer *et al.*, 2005), but are more persistent in human skin *in vivo* because of slower repair than CC (Bykov *et al.*, 1999; Xu *et al.*, 2000). However, *in vitro* studies have shown that UVA1 readily induces C→T transitions, widely associated with UVB-induced CPDs (Kappes *et al.*, 2006; Runger and Kappes, 2008), which is probably the result of UVA1-induced cytosine-containing CPDs (Mouret *et al.*, 2006).

An *ex vivo* study found that UVA1-induced TTs are repaired less well than those induced by UVB (Mouret *et al.*, 2006), as with our study there were about four times more TTs with UVB compared with UVA1. Our repair kinetics data show that this is not the case *in vivo*. UVA1-induced TTs were repaired at the same rate as those induced by UVB (Figure 4a). As UVA1 produced fewer CPDs than UVB, repair was almost complete after 48 hours. These data suggest that the degree of initial DNA damage, from an acute exposure, determines the amount of damage that remains a day or two later. However, *in vitro* studies suggest that the rate of CPD repair from a single exposure may decrease with higher doses, possibly resulting from damage to the DNA repair machinery (Greinert *et al.*, 2000; Courdavault *et al.*, 2004). The situation may be different with repeated daily sub-erythemal exposure, which results in the accumulation of TTs (Young *et al.*, 2007). Limited evidence exists that repeated sub-erythemal exposure enhances TT repair in skin types III/IV but not in I/II (Sheehan *et al.*, 2002). In contrast, mice exposed to chronic low-dose UVB show decreased CPD repair in response to a challenge dose (Mitchell *et al.*, 1999).

The photochemical mechanism by which UVA1 selectively induces TTs remains unknown. UVA activation of some photosensitizers such as fluoroquinolones (Makinen *et al.*, 1997) and carprofen (Robinson *et al.*, 2010) can induce CPDs *in vitro*, suggesting that unidentified endogenous photosensitizers might have a role in the induction of TTs by UVA1. However, double-stranded base sequences (e.g., dA.dT) increase UVA absorption compared with the same monomeric molecules (Mouret *et al.*, 2010) and UVA induction of TTs seems to be due to a direct photochemical mechanism without involvement of a cellular photosensitizer (Jiang *et al.*, 2009; Mouret *et al.*, 2010). The lack of 6-4PPs with UVA suggests that the photochemical process is different from that with UVB and UVC; however, this remains to be elucidated.

It is intriguing that UVB predominantly induced TTs in the superficial epidermis, whereas UVA1-induced TTs were more prominent in the lower epidermis (Figure 2). A similar depth effect has been found in staining for p53 protein expression at 24 hours (Campbell *et al.*, 1993) with UVB (300 ± 5 nm) and UVA (350 ± 30 nm). There are very few data on the optical properties of skin in the UVR region. Such photons may be absorbed by chromophores (e.g., DNA) or scattered by the epidermis and dermis in a forward or backward direction, and scattering may be considered more important than absorption (Anderson and Parrish, 1981; van Gemert *et al.*, 1989). Overall, skin chromophores absorb primarily in the UVB region. Back scattering results in remittance (a type of ''reflection''), which provides additional opportunity for chromophore absorption during the return pathway. Dermal remittance increases between 300 and 400 nm (Anderson and Parrish, 1981). Thus, it is possible that the higher number of UVA1-induced T<>T seen in the basal layer is due to dermal back scatter (e.g., from collagen), as well as epidermal forward scatter (Bruls and van der Leun, 1984). Irrespective of any mutagenic potential for TTs, our data suggest that UVA1 preferentially targets the stem cell-containing basal layer. This may be significant for skin cancer because more UVA fingerprint mutations are found in the basal layer compared with the suprabasal layer in squamous cell carcinomas and actinic keratoses (Agar *et al.*, 2004). Furthermore, studies on engineered human skin show that, in contrast to UVB, UVA-induced mutations were mainly located in the basal layer (Huang *et al.*, 2009).

In summary, we have demonstrated that erythemally equivalent doses of UVA1 and UVB induce TTs in human buttock skin *in vivo*, but that 6-4PPs are induced by UVB only. UVB induced more TTs than UVA1 for a given erythemal exposure. This, along with the lack of correlation between UVB and UVA1 MED, adds support to the hypothesis that the chromophores and the mechanism for UVB and UVA1 erythema are different, although presumably TTs contribute to UVA1 erythema. Our data suggest that the basal layer is particularly vulnerable to UVA1-induced damage and this is supported by some mutagenesis studies. This has implications for public health policies, particularly the need for broader waveband population photoprotection.

MATERIALS AND METHODS

Irradiation

UVR sources and dosimetry. Emission spectra and irradiances of the UVA1 and UVB sources were determined with a DM150BC double-monochromator spectroradiometer (Bentham Instruments, Reading, UK) using an integration sphere and gratings blazed at 250 nm (see Figure 5). The UVA1 source was a Sellamed 3000 Dr Sellmeier (Sellas, Gevelsberg, Germany) irradiation device. Irradiance was routinely measured with a radiometer (Model IL1400A, International Light Technologies, Peabody, MA), after calibration against the spectroradiometric measurements, and was typically about 74 mW cm^{-2} at the skin surface, which was 24.5 cm from the source. Narrowband UVB (300 nm) was produced by a monochromator (Oriel, Irvine, CA: 1-kW xenon arc; grating blazed at 250 nm; slits set for 3 nm full width at half maximum bandwidth). UVB was delivered with a liquid light guide (Oriel), with an exit diameter of 5 mm, in direct contact with the skin. Irradiance

($\sim$0.5 mW cm^{-2}) was measured with an SEL623 thermopile (International Light Technologies) attached to an IL1400A radiometer (details as above; detector and meter calibrated by the United Kingdom Accreditation Service-accredited Guy's and St Thomas' Hospitals Trust UVR laboratory).

Volunteers. The studies were approved by the St Thomas' Hospital, London, UK, Ethics Committee (ref: 09/H0802/98), and conducted in accordance with the Declaration of Helsinki Principles. The details of the 12 healthy skin type I/II (Fitzpatrick, 1988) volunteers are shown in Table 1. Participants gave written informed consent before taking part in the study.

Irradiation protocol. A geometric ($\times$1.25) series of eight doses was given over 1 cm^2 areas of previously unexposed buttock skin.

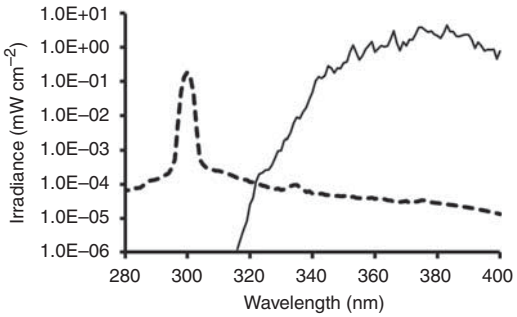

Figure 5. Emission spectra of UVB (300 nm) and UVA1 sources. Note the very clean separation of the spectra with 3–4 orders of magnitude difference between peaks and spectral crossover. The UVA2 (230–340 nm) content of the UVA1 source was 0.2%, which is equivalent to 0.6% of the overall erythemally effective energy when the emission spectrum is weighted with the action spectrum for erythema (CIE, 1998).

A visual assessment of the exposed sites was made 24 hours after exposure and the ''just perceptible MED'' was determined, a more reliable threshold end point than ''erythema with a definite border'' (Quinn *et al.*, 1994). Quantitative reflective spectroscopy to quantify erythema (erythema meter, Dia-Stron, Andover, UK) was carried out three times per test site per volunteer, and the mean of the three measurements calculated. The difference between the measurement from the test site and that from an adjacent nonirradiated control site is the Δ erythema index value.

MEDs were measured in three individuals: mean values 30.0 (SD $\pm$0) mJ cm^{-2} for UVB and 48.8 (SD $\pm$0) J cm^{-2} for UVA1. These were defined as the ''notional MED'' subsequently given to each volunteer. Table 1 shows that the mean MED for 12 volunteers were 25.8 $\pm$ 4.6 mJ cm^{-2} (UVB) and 45.8 $\pm$ 9.4 J cm^{-2} (UVA1): the notional MEDs are within one SD of these values. Punch biopsies (4 mm) were taken under local anesthesia in two studies: (i) ''dose response'': six participants were given 0.5, 1.5, and 3 notional MEDs of UVB and UVA1 and biopsies taken immediately afterward; (ii) ''time course'': six participants were given three notional MEDs of UVA1 and UVB and seven biopsies then taken at three time points, per spectrum, from each volunteer within the ranges immediate, 3, 6, 24, and 48 hours. Times varied because of ethical considerations: all had biopsies at 0 and 24 hours, except for two UVB sites on two volunteers. A nonirradiated control biopsy was taken from all volunteers.

Immunostaining. Biopsies were fixed in 10% formalin overnight ($\sim$16 hours), embedded in paraffin, and sectioned at 4 μm. Sections were deparaffinized with xylene and incubated with 0.1% trypsin (Gibco, Paisley, UK) at 37 °C for 30 minutes; slides were washed twice in phosphate-buffered saline (PBS) and incubated in 0.6% H_2O_2 and 0.1% triton X-100 in PBS for 10 minutes. After washing in tap water, DNA was denatured with 70 mM NaOH in 70% ethanol for 4 minutes.

Table 1. Volunteer demographics and their just perceptible MED

Study	Skin type	Sex	Age (years)	MED UVA1 (J cm^{-2})	MED UVB (mJ cm^{-2})
Dose response	I	F	28	48.8	30.0
	I	F	25	48.8	30.0
	II	M	20	48.8	30.0
	II	F	21	53.0	30.0
	I	F	22	48.8	23.0
	I	M	34	61.1	19.0
Mean $\pm$ SD		M+F	25 $\pm$ 5.3	51.6 $\pm$ 4.9	27.0 $\pm$ 4.8
Time course	I	F	28	31.3	19
	I	F	28	31.3	30
	I	M	22	48.8	23
	II	F	24	31.3	30
	I	M	23	48.8	23
	II	F	23	48.8	23
Mean $\pm$ SD		M+F	24.7 $\pm$ 2.7	40.1 $\pm$ 9.6	24.7 $\pm$ 4.4
Combined	I (8), II (4)	M (4), F (8)	24.8 $\pm$ 4.0	45.8 $\pm$ 9.4	25.8 $\pm$ 4.6

Abbreviations: F, female; M, male; MED, minimal erythema dose.

Slides were washed twice with PBS and incubated for 20 minutes in blocking buffer (10% goat serum, 0.1% bovine serum albumin, and 0.1% Tween-20 in PBS), followed by monoclonal antibody incubation for 90 minutes at room temperature. The antibodies were TDM-2 (CosmoBio, Tokyo, Japan) at 1:2,000, which has primary specificity to TTs but also can recognize other CPDs (Mori *et al.*, 1991), and 64M2 (CosmoBio) at 1:300, which has primary specificity to TT 6-4PPs but also recognizes other 6-4PPs (Mori *et al.*, 1991). In addition, an isotype control IgG2a (DAKO, Cambridge, UK) was used. After washing twice in PBS, slides were incubated with Alexa Fluor goat anti-mouse 555 (Invitrogen, Paisley, UK) at 1:200 for 30 minutes, counterstained with prolong gold antifade with DAPI (Molecular Probes, Paisley, UK), coverslipped, and stored away from light.

Isotype controls, to exclude nonspecific antibody staining, were negative in every experiment. The reproducibility of staining and image analysis was measured for both the dose–response and time-course studies. Thus, three notional MEDs of UVB or UVA1 (taken immediately after exposure) were stained with each staining run within both experiments. Mean nuclear intensities for TTs were (mean ± SD) 117.8 ± 7.8 for UVB and 22.4 ± 6.6 for UVA1 (dose response), and 99.6 ± 3.1 for UVB and 36.7 ± 3.1 for UVA1 (time response). For 6-4PPs, the comparable values for the dose response were 29.8 ± 14.2 for UVB and 0 ± 0 for UVA1, and for the time response were 34.9 ± 14.1 for UVB and 0 ± 0 for UVA1. These data indicate that the staining and imaging analysis techniques were reproducible within a given experiment.

CPD and 6-4PP quantification by immunofluorescence image analysis. Fluorescent imaging was done for Alexa Fluor 555 (red) and DAPI (nuclear DNA stain; blue): Zeiss Axiophot microscope (Harpenden, UK) and Nikon DS-U2 camera (Kingston upon Thames, UK) with × 20 magnification (and × 1.25 eyepiece objective). Images were acquired in 2,560 × 1,920 format and exposed to 20S (gain × 1.40 for Alexa Fluor5 55 with 1.5 seconds exposure, gain × 1.40 for DAPI). Contrast setting was the same for all images. Epidermis analysis was done using NIS elements BR v3 software package (Melville, NY). DAPI-stained nuclei were gated and mean red intensity (Alexa Fluor 555) assessed within each of at least 200 nuclei. Mean background intensity from the nonirradiated control also calculated for over 200 nuclei and subtracted from the irradiated samples to control for nonspecific nuclear staining (typically higher for 6-4PPs: 44.9 ± 36.7 than TTs: 12.6 ± 6.4 as previously reported (Young *et al.*, 1996)). Settings for nuclei capture (circularity and diameter of nuclei) were maintained constant, sections subjectively assessed, and nuclei ''separated and smoothed'' as necessary. For each biopsy, the mean intensity value for nuclei in the epidermis was calculated.

Quantification of the distribution of epidermal TT staining. The ''3MED'' UVA1 and UVB biopsies, 0 hour, from 12 volunteers were assessed for nuclear staining intensity on a line perpendicular to the surface over the thickest section of the epidermis. DAPI-stained epidermal nuclei were gated and red staining of each nucleus on the line was individually gated and measured.

Reproducibility of staining and image analysis. It was not possible to stain and assess all slides in a single batch. To assess the reproducibility of the techniques, sections prepared from one individual exposed to three notional MED UVB and UVA1 were processed with each staining and image analysis run, as well as a nonirradiated control and an isotype control in each case.

Data analysis

Linear regression analysis was used to study correlation between UVR dose and outcome. Statistical tests used Graphpad Prism v4 statistics package (La Jolla, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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CASE REPORT

BJD
British Journal of Dermatology

A case of extensive hyaline deposition in facial skin caused by erythropoietic protoporphyria

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Summary

Although erythropoietic protoporphyria (EPP) is relatively uncommon, affecting approximately 1 in 140 000 individuals in the U.K., it is an important disease not to miss owing to the risk of acute severe liver disease in 2% of cases. EPP occurs with clinical and histological changes in the skin associated with free-radical-associated dermal vascular damage. This also mediates the painful photosensitivity. Severe and disfiguring hyaline deposition is extremely rare. We demonstrate that severe EPP can cause disfiguring hyaline infiltration of the skin on the hands and face, which sheds light on the mechanism of photosensitivity in EPP; it must also be differentiated from conditions such as lipoid proteinosis.

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What's already known about this topic?

- Erythropoietic protoporphyria (EPP) is an important disease not to miss owing to the risk of acute severe liver disease in 2% of cases.
- Severe and disfiguring hyaline deposition is extremely rare.

What does this study add?

- We show how severe EPP can cause disfiguring hyaline infiltration of the skin on the hands and face.
- This must be differentiated from conditions such as lipoid proteinosis.

Case report

A 59-year-old woman presented with lifelong severe photosensitivity due to erythropoietic protoporphyria (EPP). From early childhood she had suffered bouts of burning pain affecting the skin of the face, neck and hands. The bouts were triggered by 3 min of exposure to summer sunshine, and were associated with visible swelling that lasted 2–3 days. The diagnosis of EPP was confirmed by an increased erythrocyte-free protoporphyrin concentration of 59 μmol L^{-1} (normal range $< 0{\cdot}2$). Over the previous 2 years there had been progressive disfiguring, furrowing and thickening of the skin of the face and hands (Fig. 1). Histology of a forehead skin biopsy revealed deposits of amorphous eosinophilic material throughout the papillary and mid-dermis, centred around and compressing dermal capillaries (Fig. 2). Periodic acid–Schiff (PAS) staining of this hyaline material was positive, but amyloid stains (Congo red and thioflavin T) were negative. This pattern of hyaline deposition is not seen in lipoid proteinosis, in which there is additional involvement of eccrine glands or colloid milium, which has a characteristic fissured appearance on histology. A diagnosis of lipoid proteinosis was excluded from the clinical history as there was an absence of symptoms manifesting in childhood, such as a hoarse voice, hair loss or neuropsychiatric symptoms. The disfigurement was found solely on photo-exposed skin, and there was no evidence of moniliform blepharosis or plaques at any trauma sites.

Normally, exposed skin in EPP contains small amounts of PAS-positive eosinophilic deposits of hyaline in a perivascular distribution in the upper dermis.[1] Clinically, this is reflected in the classical findings of a waxy appearance of facial skin, perioral radial fine linear scars, fine linear and crateriform scarring on the cheeks, and thickening of the skin over the

Fig 1. Extensive thickening and furrowing of facial skin secondary to dermal hyaline infiltration in a patient with erythropoietic protoporphyria.

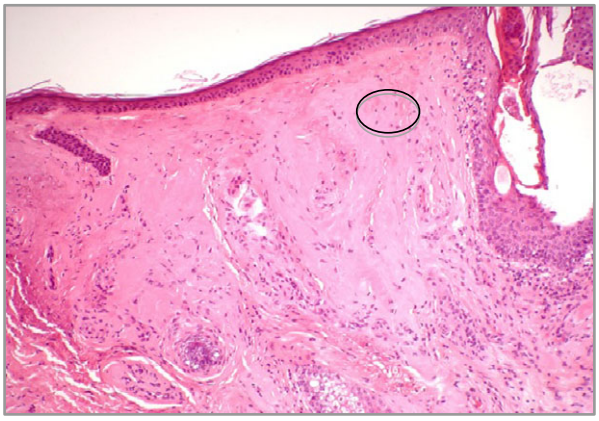

Fig 2. Forehead skin biopsy showing amorphous eosinophilic material accumulating in the papillary dermis (circled). This is far more extensive than the accumulation usually seen in erythropoietic protoporphyria.

knuckles.[2] In EPP, these hyaline deposits have been shown on immunohistochemistry and electron microscopy to contain plasma proteins, including fibrinogen and immunoglobulins.[1] This reflects the pathogenesis of the painful photosensitivity in EPP, which involves free-radical-mediated necrosis of upper dermal small blood vessel endothelium, with hyaline accumulating as a result of repeated episodes of damage and plasma leaking through vessel walls into the surrounding dermal tissue.[3] The resulting regeneration of the basement membrane causes the characteristic reduplication seen on electron microscopy.[1]

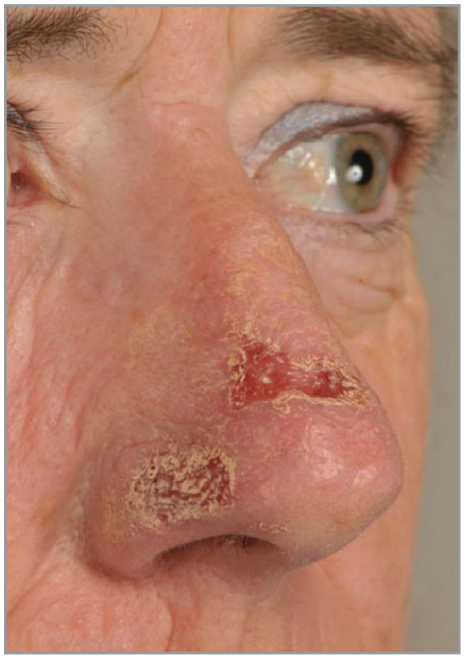

Fig 3. Crusts on the malar and right lateral aspect of the nose during a severe bout of photosensitivity in our patient.

The exaggerated, extensive and clinically disfiguring hyaline deposition seen in this patient is unusual. Clinically, it resembles lipoid proteinosis. We postulate that the extensive deposition of hyaline reflects the severe nature of the patient's EPP photosensitivity; 3–4 min of U.K. summer sun is an unusually small dose of light to trigger a bout of pain in EPP.[2] The severity of the photosensitivity is also clear from the repeated formation of crusts (Fig. 3), a classical feature of severe bouts of EPP photosensitivity. Although EPP has been recognized in large numbers of patients worldwide since it was first described in 1961,[4] this type of disfigurement and extensive deposition of hyaline in the skin has been described in only five patients.[5,6] Therapeutically, options are limited and patient education on photoprotection from visible light is important. In this patient, despite sun avoidance strategies and the use of photoprotection, there is still extensive skin disfigurement. The hyaline deposits are too deep for resurfacing procedures to be carried out without causing scarring. In an attempt to reduce the prominence of the furrowing, the patient is currently being treated with dermal hyaluronic acid injections. This rare and disfiguring complication of EPP highlights the pathogenetic mechanism of photosensitivity in EPP.

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RESEARCH LETTERS

Oral cholestyramine is not an effective treatment for uncomplicated erythropoietic protoporphyria

To the Editor: Erythropoietic protoporphyria (EPP) is an inherited disease in which protoporphyrin accumulates in erythrocytes, causing lifelong painful photosensitivity. There is currently no effective treatment.

One therapy that has been reported in isolated cases of EPP is the use of cholestyramine. This anion exchange resin binds to porphyrins and has been shown to disrupt enterohepatic recirculation of bile acids.[1] It has been suggested that cholestyramine might increase biliary excretion of protoporphyrin by interrupting its enterohepatic recirculation.[1] In one patient with EPP,[1] cholestyramine increased fecal excretion of the porphyrin, and although there was concurrent improvement in liver function tests, these are isolated reports. Unexpectedly, it has also been observed to increase urine porphyrin excretion in 3 patients with EPP.[2] Activated charcoal, an alternative porphyrin chelator, has also been tried in EPP,[3] although it has no effect in patients with congenital erythropoietic porphyria(CEP)[4] and causes a paradoxical clinical and biochemical deterioration in variegate porphyria.[5]

The absence of effective treatment for EPP makes it important to establish whether a treatment such as cholestyramine is effective or not. Since red cell and plasma protoporphyrin concentrations are the pathologically relevant measures, we assayed these in 3 EPP patients before, during, and after treatment with oral cholestyramine. We chose to study 3 adult patients with EPP (with classic and severe photosensitivity from childhood, and without evidence of liver dysfunction) as this number gives statistical significance to our data.

The study was conducted in compliance with the principles of the Declaration of Helsinki under the regulation of the Medicines and Healthcare products Regulatory Agency. Red cell and plasma protoporphyrin were measured monthly during the 3 months of therapy with cholestyramine (12 grams daily) and for 2 months before and after the treatment. Compliance cannot be objectively assessed, but we suspect that patient 2 may not have been fully compliant with the therapy.

Fig 1 shows the data for the 3 patients. The overall impression is that the blood protoporphyrin concentrations are unaffected by treatment and this is confirmed using the nonparametric Skillings-Mack test ($P = .29$, $P = .40$ for red cell and plasma porphyrins, respectively) and there is no significant difference between the values on and off the therapy. The patients also showed no change in clinical symptoms throughout the study time period.

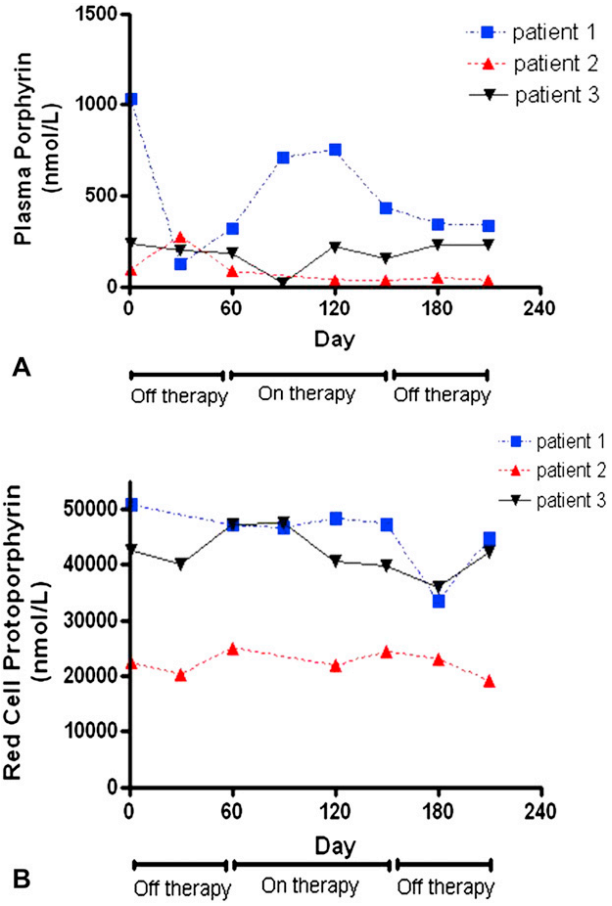

Fig 1. A, Plasma porphyrin levels for each of the 3 patients. **B,** Red cell protoporphyrin levels for each of the 3 patients.

Cholestyramine does not reduce blood protoporphyrin IX concentrations in uncomplicated EPP and is therefore not an effective therapy for patients with EPP. It has been proposed that cholestyramine may be of value in protecting the liver in protoporphyric liver disease, a hypothesis not assessed in this study.

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The current state of dermatology training: A national survey of graduating dermatology residents

To the Editor: The education of residents is a dynamic and challenging process that has greatly evolved in the last several years. The comprehensive dermatology residency experience from the residents' perspective has not been described since 1996.[1] Since this time, elemental shifts have morphed the once apprentice-style training into a more formal education process. Given the diversity of approaches to resident training, we conducted a survey of graduating dermatology residents to assess the current state of the dermatology residency experience.

A 53-question Web-based survey (www.kwiksurveys.com, Dover, United Kingdom) was offered to graduating resident physicians at all 109 Accreditation Council for Graduate Medical Education (ACGME)–approved dermatology residency programs in June 2011. To assess resident comfort levels with medical, surgical, and pediatric dermatology and histopathology, a 5-level Likert scale was used. Statistical analyses were performed using chi-square tests for categorical variables and Spearman's rank correlation for ordinal variables. All tests were two-tailed, and P values less than or equal to .05 were considered significant.

A total of 127 surveys (39%) were received. Responding residents were primarily female (61%) with an average age of 32 $\pm$ 3 years. The majority (53%) of residents reported an average program size of 7 to 12 total residents, with 70% having 6 to 15 full-time attendings. Sixty-two percent of survey participants described their clinics as "attending-run" (clinics in which attendings have primary responsibility over care). No associations were found between the type of clinic and the level of anxiety of beginning one's career, stress level during residency, feelings of 'burnt out', and level of adequacy of training. Residents, on average, attended 8 $\pm$ 1 half-day clinics per week, with a median of 1 pediatrics clinic per week, and saw 7 to 12 patients per clinic. Higher levels of feeling adequately trained among residents were associated with working more clinics per week and seeing more patients per clinic ($P = .02$ and $P = .05$, respectively). Overall, 75% of respondents felt autonomous in regards to patient care as third-year residents.

Regarding lectures, 81% of respondents had none to 3 faculty-led lectures per week and 59% had none to 3 resident-led lectures per week. Forty-six percent had a mandatory research requirement, 38% had a formal mentorship program, and 84% had a continuity clinic. Sixty-three percent studied 4 to 9 hours outside of clinic per week and, on average, had 1 full day of academic time per week. Program characteristics are summarized in Table I.

Regarding comfort and competency with medical dermatology, 93% felt somewhat or very comfortable prescribing medications that require lab monitoring, 89% with prescribing biologics, 80% with phototherapy, and 43% with infusions. Regarding surgical dermatology, 98% felt somewhat or very comfortable performing simple excisions, 45% performing flaps, 41% performing grafts, and 17% performing Mohs micrographic surgery. Results are summarized in Fig 1.

Assessing comprehensive nationwide residency curricula is difficult. Few studies have addressed this issue, and among those conducted, the results are limited in scope.[1-5] Interestingly, despite changes in

Successful treatment of microstomia with UVA1 phototherapy in systemic sclerosis

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Key words:
UVA1; microstomia; systemic sclerosis

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To the Editor,

The introduction of phototherapy has substantially changed the therapeutic response of localized scleroderma (LS). Systemic agents have been proposed for the treatment of LS, some with potential side effects and varying degrees of success. There is sufficient evidence in the literature to demonstrate that low dose ($20\,J/cm^2$) and medium dose ($50\,J/cm^2$) UVA1 phototherapy is beneficial in LS. The development of a metal halide lamp emitting high levels of UVA1 radiation (340–400 nm) was first described in 1981 (1), but the therapeutic potential for UVA1 phototherapy did not emerge until 1992 with the first successful report of treating patients for acute exacerbations of atopic dermatitis. It has now been shown to soften thickened plaques, increase skin elasticity and reduce lesional skin thickness in scleroderma (2–4). Recently (4), low-dose UVA1, medium-dose UVA1 and narrowband UVB phototherapy were compared demonstrating comparable efficacy of narrowband UVB and low-dose UVA1 but medium-dose UVA1 being more effective. In systemic sclerosis (SSc), studies of UVA1 are limited. Improvement has been documented in acrosclerosis in those patients with SSc (5). There is no documentation of treatment of other features of SSc by phototherapy in the literature. We describe a case of successful treatment of microstomia with UVA1 phototherapy in a patient with SSc.

Case report

A 45-year-old woman with SCL70+ve systemic sclerosis presented with symptomatic sclerodactyly and microstomia, which had been progressively worsening over the last 5 years. Previous treatments included hydroxychlorquine and she had declined systemic corticosteroids and other systemic immunosuppressant therapy. There was no relevant past medical history or family history and she worked as a solicitor.

On examination, there was evidence of significantly tight, thick skin affecting the hands, face, arms and upper chest and microstomia. She found it difficult to open her mouth and articulate her words and there was a slight pinching of her nose. SCL 70 antibodies were present in peripheral blood and other blood tests including inflammatory markers, FBC, urea/creatinine, LFTs and complement were normal. She was commenced on $50\,J/cm^2$ UVA1 phototherapy to the whole skin surface 2–3 times weekly. After 10 treatments all the sclerotic areas of skin including the perioral area were less indurated on examination. After 21 treatments she could articulate words normally and there was reduced furrowing around the mouth. After 40 treatments and a total of $2225\,J/cm^2$ she could open her mouth more normally: the anterior–posterior diameter from the lower margin of the front teeth to the upper margin of the lower teeth had increased by 1 cm (25%) from 4 to 5 cm (Figs 1 and 2).

Fig. 1. March 2009.

Fig. 2. April 2010.

Currently she says that brushing her teeth, eating and talking have all become significantly easier following the treatments.

Discussion

Equipment to deliver UVA1 (340–400 nm) waveband has been available from 1981 but it is only in the last 20 years that increasing beneficial use of UVA1 has been documented (4). UVA1 is accepted effective treatment for morphoea(2–4), systemic scleroderma (3) and granulomatous chronic GVHD (3). It is also used and may be effective in urticaria pigmentosa (3, 4), scleredema adultorum Bushke (3, 4), granuloma annulare (4), nodular prurigo (4), mycosis fungoides (3), atopic eczema (4), polymorphic light eruption (4) and SLE (3).

Systemic sclerosis is difficult to treat. Despite advances in disease-specific treatment of other rheumatologic diseases, disease-targeted treatment in systemic sclerosis continues to be elusive suggesting treatment involves a complex interaction of specific targets. There are no published studies of treatment of microstomia in systemic sclerosis. UVA1 phototherapy exerts its therapeutic effects through modulation of three predominant pathogenic mechanisms in sclerosis: immune dysregulation, imbalance of collagen deposition and endothelial dysfunction (5). In our case, UVA1 appears to have been effective in softening sclerotic perioral skin and improving symptoms caused by the microstomia. This case highlights how UVA1 phototherapy should be considered early if patients with systemic sclerosis and significant disability with skin involvement such as microstomia are unable to tolerate systemic therapy, but also importantly as an early adjunct to systemic therapy.

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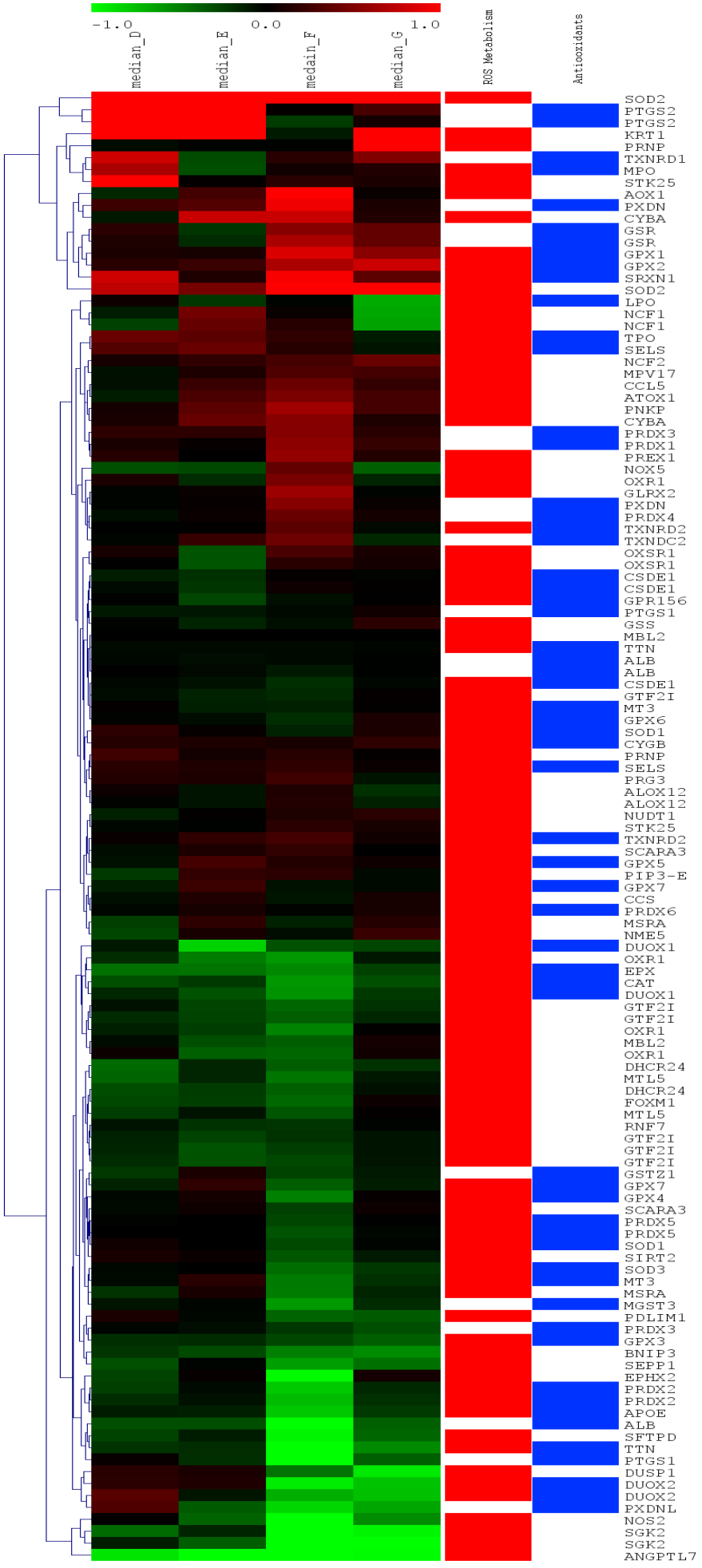
Appendix C

Heat map showing gene expression profiles of UVA1 and UVB at 6h (D, E) and 24h (G, F) for selected genes with antioxidant activity

This figure shows hierarchical clustering of genes statistically significant ($p<0.05$) for D UVA1 6h, E UVB 6h, F UVB 24h and G UVA1 24h. Color saturation limits were set in log2 scale from -1 (bright green) to +1 (bright red). Genes associated with ROS metabolism and antioxidant activity are indicated by red and blue labels next to the gene name, respectively.

This map shows intense differential expression predominantly at 6h, although there is still strong expression by UVB at 24h (F). There is also a large degree of spread with some genes upregulated by UVB and others by UVA1.

Appendix C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ROS Metabolism
Antioxidants
SOD2
PTGS2
PTGS2
KRT1
PRNP
TXNRD1
MPO
STK25
AOX1
PXDN
CYBA
GSR
GSR
GPX1
GPX2
SRXN1
SOD2
LPO
NCF1
NCF1
TPO
SELS
NCF2
MPV17
CCL5
ATOX1
PNKP
CYBA
PRDX3
PRDX1
PREX1
NOX5
OXR1
GLRX2
PXDN
PRDX4
TXNRD2
TXNDC2
OXSR1
OXSR1
CSDE1
CSDE1
GPR156
PTGS1
GSS
MBL2
TTN
ALB
ALB
CSDE1
GTF2I
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GPX6
SOD1
CYGB
PRNP
SELS
PRG3
ALOX12
ALOX12
NUDT1
STK25
TXNRD2
SCARA3
GPX5
PIP3-E
GPX7
CCS
PRDX6
MSRA
NME5
DUOX1
OXR1
EPX
CAT
DUOX1
GTF2I
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OXR1
MBL2
OXR1
DHCR24
MTL5
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FOXM1
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GSTZ1
GPX7
GPX4
SCARA3
PRDX5
PRDX5
SOD1
SIRT2
SOD3
MT3
MSRA
MGST3
PDLIM1
PRDX3
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PRDX2
PRDX2
APOE
ALB
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DUOX2
DUOX2
PXDNL
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SGK2
SGK2
ANGPTL7